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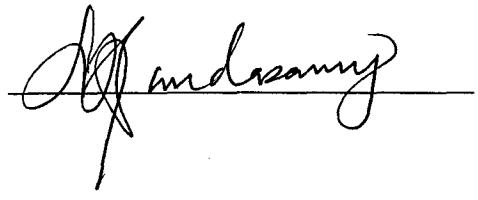

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13. ABSTRACT *(Maximum 200 words)*

These studies focus on mechanisms by which the E1A oncogene sensitizes tumor cells to destruction by cytolytic lymphocytes and other injuries. The preliminary observation that E1A sensitizes cells to proapoptotic injuries, but not injuries that trigger cellular necrosis has been confirmed. Our speculation that E1A functions independently of the p53 transcription factor has been confirmed. Other studies showed that co-expression of the Bcl-2-like molecule, E1B 19 kD, does not block the E1A sensitizing effect. These data further focus the proposed Tasks. Thus, the search for E1A-modulated cellular genes whose altered expression renders cells sensitive to cytolytic injury (Task 1) can now be focused on a p53-independent apoptosis pathway that is not blocked by Bcl-2 activity. RHKO mutagenesis, differential display and PCR-based subtractive hybridization studies have provided limited information on E1A modulation of cellular genes and are being replaced by plans for studies using cDNA chip technology.

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FOREWORD

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James S. Cook MD 7/26/99

PI - Signature Date

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INTRODUCTION:

These studies exploit our observation that expression of the E1A oncogene of human adenovirus types 2 and 5 converts formerly resistant cells into cells that are highly sensitive to lysis induced by injury by killer lymphocytes, the cytokine TNF alpha and a variety of chemical agents, including those used for cancer chemotherapy (**cytolytic susceptibility**). The purpose of the proposed experiments is to better understand the cellular pathways through which this E1A activity is mediated, with the long-term goal of developing methods to therapeutically trigger this type of sensitizing response in breast cancer cells. Preliminary observations have been pursued to narrow the scope of these studies to more clearly defined molecular pathways and to define the best methods for identifying the key cellular genes whose E1A modulated expression is involved in this oncogene-induced change in tumor cell phenotype.

BODY:

Task 1 was to identify cellular genes whose expression is modulated by E1A to cause increased cytolytic susceptibility. Preliminary observations were presented in the original application suggesting that the cellular apoptosis pathway was involved in this E1A activity. Formal testing of this hypothesis has been completed, and the results have been accepted for publication (1, Appendix A). In those studies, we asked whether E1A sensitization to injury-induced apoptosis was sufficient to explain E1A-induced cytolytic susceptibility or whether an injury-induced necrotic response was also involved. Tumor cells that were converted to the cytolytic susceptible phenotype by E1A expression were also found to be sensitized to CL-induced and chemically-induced apoptosis, but were no more susceptible to injury-induced necrosis than E1A-negative cells. Similar to induction of cytolytic susceptibility and in contrast to other E1A gene activities, sensitization to chemically-induced apoptosis depended on high level, E1A oncoprotein expression. Loss of both cytolytic susceptibility and sensitization to chemically-induced apoptosis were co-selected during *in vivo* selection of E1A-positive sarcoma cells for increased tumorigenicity. Furthermore, E1A gene mutations that abrogated cytolytic susceptibility also eliminated E1A sensitization of cells to injury-induced apoptosis. These data indicated that E1A induces susceptibility to killer cell-induced lysis through an apoptotic pathway and that the cellular necrotic response was not involved. These data narrowed the search for cellular pathways and genes through which E1A induces cytolytic susceptibility to those involved in apoptosis.

A second preliminary observation that was presented in the original proposal and that is related to the Task 1 was that cellular expression of the p53 transcriptional activator may not be required for E1A-induced cytolytic susceptibility. This hypothesis was tested by contrasting the ability of E1A oncoprotein expression to convert cytolytic resistant tumor cells a the cytolytic susceptible (and apoptosis sensitive) state in the presence and absence of cellular p53 expression. These data have been submitted for publication (2, Appendix B). Cytolytic lymphocytes (CL), TNF alpha and a variety of chemical injuries were used to test these cells. The results showed that E1A sensitization to immune-mediated (CL- or TNF-induced) apoptosis was independent of p53 expression. In contrast, the p53 requirement for chemically induced apoptosis of E1A-sensitized cells varied with the agent used to treat cells. Thus, the protein synthesis inhibitor, hygromycin, triggered apoptosis in E1A-positive cells, independently of p53 expression, whereas triggering of

apoptosis of E1A-positive cells by three other chemical agents (beauvericin, etoposide and hydrogen peroxide) was strictly dependent on p53 expression. E1A-positive but p53-negative cells that were fully sensitive to immune-mediated apoptosis were highly resistant to apoptosis triggered by the latter three agents. We interpret these data to indicate that the basic cellular mechanism(s) through which E1A sensitizes cells to apoptotic injury do not require p53. However, the mechanisms by which different agents trigger the apoptotic response can be highly dependent on p53. These observations narrowed the search for the fundamental mechanism(s) of E1A-induced sensitization to apoptosis to one or more apoptosis pathways that do not require p53 expression.

A third preliminary observation was presented in the original proposal that bridges Tasks 1 and 2. In Task 2, we proposed to study the interactions between E1A oncogene expression (now known to sensitize cells to proapoptotic injuries) and Bcl-2-like cellular activities that can block certain apoptotic pathways. These studies had the advantage of testing the role of the Bcl-2 blocking mechanism (Task 2) while also providing more focus to the definition of the relevant E1A-controlled cellular pathways and genes proposed in Task 1.

It was known that E1A-related, p53-*dependent* apoptosis could be blocked by co-expression of the adenoviral E1B 19 kD protein. E1B 19 kD is a member of the family of viral and cellular proteins that inhibit apoptosis, with Bcl-2 being the prototype member (reviewed in 3). E1B 19 kD, like Bcl-2, can block some types of proapoptotic injuries, but not others. We had reported that E1B expression did not prevent E1A induction of cellular susceptibility to lysis by cytolytic lymphocytes or activated macrophages (4, 5, 6, 7, 8, 9, 10, 11). Studies were done here to test the prediction that E1B 19 kD would not block CL- or TNF-induced apoptosis and to contrast the E1B effects on immune-mediated apoptosis with effects against various proapoptotic, chemical agents. Apoptotic sensitivities of cells were contrasted using CL, TNF, hygromycin, beauvericin, etoposide and H_20_2. E1B 19 kD had no significant inhibitory effect on apoptosis induced by either CL or TNF alpha. However, E1B 19 kD blocked E1A-induced sensitization to apoptosis induced by hygromycin, beauvericin, etoposide and H_20_2. The ability of E1B 19 kD to block E1A sensitization to apoptosis was not linked to the p53-dependence of the injury, since hygromycin (a p53-independent injury) and all three p53-dependent injuries (beauvericin, etoposide and H_20_2) were blocked, but CL and TNF (both p53-independent injuries) were not. These data have been submitted for publication (2, Appendix B). These results narrowed the focus of studies of the cellular pathways through which E1A sensitizes tumor cells to immune-mediated apoptosis to mechanisms that are both p53-independent and resistant to blockade by Bcl-2-likemechanisms.

Preliminary studies using the method of random homozygous knockout (RHKO) mutagenesis of allelic loci (12) that were proposed in Task 1 have not identified differentially expressed genes in E1A-positive vs. E1A-negative cells. Therefore, effort has been focused on studies of a modification of mRNA differential display (13) and PCR-based subtractive hybridization (Clontech, PCR-Select). We also plan studies using the recently available and powerful cDNA chip method to assess E1A-induced differential gene expression in breast cancer cells. The objective of these studies is to obtain genetic information to complement the biological data presented above in analysis of the molecular mechanisms by which E1A expression to sensitizes cells to apoptotic injury.

The differential display method has identified two cellular genes to date that are repressed in E1A-positive cells when contrasted with E1A-negative cells and that will be considered for further study — osteopontin and ST2. The initial osteopontin data were presented in the original application and have not been pursued further at this point. The second gene that has been identified and that is differentially repressed by E1A is ST2. ST-2 is an integrin-like molecule that is an IL-1 receptor (IL-1R) family member (14). Our unpublished data indicate that E1A represses the soluble form of ST2, ST2-S. The question relevant to these studies is whether E1A repression of ST2 expression is generally observed among different human breast cancer cell lines and whether this gene repressive effect is functionally linked with E1A-induced sensitization to apoptosis. A two-staged approach is being used for further studies of the role of ST2-S in this E1A activity. We are modeling the possible mechanisms through which repression of ST2-S could increase cellular apoptosis in response to injury. We will use these concepts to develop specific experiments that test the hypotheses generated. It may be interesting that IL-1 treatment can protect certain types of cells from TNF-induced apoptosis, possibly by activating an NF-kB-dependent cellular defense (15) and that E1A expression has been reported to convert IL-1 treatment into a stimulus that enhances the cellular apoptotic response (16). These observations suggest the possibility that E1A may alter interactions between IL-1R-family molecules – such as ST2 – and their cognate ligands, consequently altering the effects of these signals on cellular responses to secondary signals. These and other possibilities will be considered and explored.

unpublished data

Discussion. These observations on E1A-induced, differential gene expression in tumor cells emphasize the importance of a two-pronged approach to the problem being studied in this project. For Task 1, it is important to determine how the effects of E1A expression on cellular gene expression be screened most effectively? It is also important, however, to have a complementary means to assess the biological relevance of any differences in gene expression between E1A-negative and E1A-positive tumor cells. We propose that it will be important to use biological assays to further define the biological nature of the E1A sensitizing effect. For example, our recently completed studies showing that the E1A mechanism of cellular sensitization to apoptotic injury does not require p53 expression and is not blocked by the Bcl-2-like activity of E1B 19 kD are useful in this regard. These are the types of studies that will continue as defined in Task 2. It will also be important to have a more efficient means of contrasting E1A-negative and E1A-positive breast cancer cells for differences in cellular gene expression to identify a variety of candidate genes to consider for these pathways. For this purpose, we plan to use the cDNA chip technology that has become increasingly available and affordable. This will allow us to expand the scope of the search for E1A-regulated genes and, perhaps equally importantly, to look for simultaneous, E1A-induced changes in multiple genes that could affect the cellular apoptosis pathways. This complementary approach is consistent with the originally proposed Statement of Work.

KEY RESEARCH ACCOMPLISHMENTS:

- Narrowed the focus of the search for the mechanisms through which E1A sensitizes cells to cytolytic injury to a p53-independent cellular pathway that is not blocked by the Bcl-2-like activity of E1B 19 kD

- Determined that certain cellular injuries (e.g., cytolytic lymphocytes, TNF alpha and hygromycin) trigger apoptosis through p53-independent mechanisms, whereas other injuries (e.g., beauvericin, etoposide, and H_20_2) require p53 expression to trigger apoptosis

- Determined that RHKO mutagenesis is not useful for the studies of E1A-induced, differential gene induction and that differential display is of limited use — decided to focus these studies on cDNA chip studies.

- Identified osteopontin and ST2 as two cellular genes whose expression is repressed by E1A

REPORTABLE OUTCOMES:

1. Cook J, Routes B, Walker T, Colvin K, Routes J. E1A oncogene induction of susceptibility to killing by cytolytic lymphocytes through target cell sensitization to apoptotic injury. Experimental Cell Research. In press, 1999.

2. Cook J, Routes B, Leu C, Walker T, Colvin K. E1A oncogene-induced cellular sensitization to immune-mediated apoptosis is independent of p53 and resistant to blockade by E1B 19 kD protein. Experimental Cell Research. Submitted for publication, 1999.

CONCLUSIONS:

The results obtained during year 1 increase the understanding of mechanisms that are influenced by the E1A oncogene to increase tumor cell susceptibility to killing by components of the antineoplastic cellular immune response. These studies indicate that this sensitizing process involves a p53-independent cellular apoptosis pathway that is not blocked Bcl-2 activity. The results of the studies of different proapoptotic injuries indicate the importance of considering the type of triggering agent being tested when assessing the p53 requirement for apoptosis. It was found that detection of E1A-induced cellular sensitization to apoptosis is highly dependent on the type of injury being tested. For studies of immune-mediated apoptosis (e.g., that triggered by cytolytic lymphocytes or TNF), p53 expression is not required.

The studies of E1A-regulated cellular gene expression demonstrated the difficulty of identifying many E1A-regulated genes using either RHKO mutagenesis or differential display. These results suggest that the use of cDNA chip technology may be more useful for a general analysis of the effects of E1A expression on the pattern of gene expression in breast cancer cells. This opportunity to assess a large array of cellular genes in the context of the available biological information on the cellular pathways through which E1A sensitizes to apoptotic injury may be highly useful for hypothesis generation in this model.

"So what?" — The long-term goal of this project is to use E1A as a genetic tool to identify molecular mechanisms that can be used to render resistant breast cancer cells much more sensitive to elimination by immunological defenses and chemotherapeutic agents. It is highly likely that progress toward this goal will require an improved definition of the cellular pathways through which cells can be converted from an "apoptosis-resistant" to an "apoptosis-sensitive" phenotype. E1A expression does this to breast cancer cells (as well as other types of mammalian cells). Since E1A is a well-known regulator of transcription, it is likely that this E1A-induced sensitizing effect is mediated by altered expression of targeted cellular genes. The objective of this project is to define one or more of these mechanisms and to use what is learned to develop strategies for novel forms of therapy that will sensitize breast cancer cells to immunotherapeutic and chemotherapeutic treatments.

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Running title: E1A sensitization to killer cell-induced apoptosis

E1A Oncogene Induction of Cellular Susceptibility to Killing by Cytolytic Lymphocytes Through Target Cell Sensitization to Apoptotic Injury

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ABSTRACT

E1A oncogene expression increases mammalian cell susceptibility to lysis by cytolytic lymphocytes (CL) at a stage in this intercellular interaction that is independent of cell surface recognition events. Since CL can induce either apoptotic or necrotic cell death, we asked whether E1A sensitization to injury-induced apoptosis is sufficient to explain E1A-induced cytolytic susceptibility. Mouse, rat, hamster and human cells that were rendered cytolytic-susceptible by E1A were also sensitized to CL-induced and chemically-induced apoptosis. In contrast, E1A-positive cells were no more susceptible to injury-induced necrosis than E1A-negative cells. Similar to induction of cytolytic susceptibility and in contrast to other E1A activities, cellular sensitization to chemically-induced apoptosis depended on high level, E1A oncoprotein expression. Loss of both cytolytic susceptibility and sensitization to chemically-induced apoptosis were co-selected during *in vivo* selection of E1A-positive sarcoma cells for increased tumorigenicity. Furthermore, E1A mutant proteins that cannot bind the cellular transcriptional coactivator, p300, that fail to induce cytolytic susceptibility also fail to sensitize cells to injury-induced apoptosis. These data indicate that E1A induces susceptibility to killer cell-induced lysis through sensitization of cells to injury-induced apoptosis.

Key words: adenovirus, E1A, oncogene, cytolytic lymphocyte, beauvericin, apoptosis, NIH-3T3

INTRODUCTION

In addition to its roles in controlling viral gene expression and the cell cycle, the E1A oncogene of human adenovirus (Ad) types 2 and 5 also actively induces cells to become highly susceptible to lysis by several components of the antitumor immune response, including different types of cytotoxic lymphocytes (CL), activated macrophages and TNF alpha [15-17, 19, 45, 55]. This E1A-induced cellular phenotype has been termed "cytolytic susceptibility." We and others proposed that E1A-induced cytolytic susceptibility explains the lack of tumorigenicity of cells transformed by these nononcogenic Ad serotypes in immunocompetent animals [8, 10, 19, 44, 45, 49, 50, 55].

We reported that the mechanism by which E1A induces the cytolytic susceptible phenotype involves a stage in the interaction between CL and their E1A-expressing target cells that follows, and is independent of, interactions between killer cell or cytokine ligands and target cell receptors — a "post-recognition" stage in cellular injury [18]. These observations suggest that E1A induces cytolytic susceptibility by causing a qualitative change in the cellular response to CL-induced injury.

Most reports indicate that CL kill their target cells by inducing apoptosis through two mechanisms — one caused by the joint actions of CL granule-associated perforin and granzymes and the other resulting from CL surface Fas-ligand cross-linking of Fas antigen on target cells [reviewed in 2, 5]. However, there is also evidence suggesting that CL-induced apoptosis may not explain all killing activity and that CL can also cause a necrotic cell death response in at least some types of target cells [20, 51, 54]. Therefore, it was not possible, using only assays of CL-induced injury, to determine whether E1A induction of cytolytic susceptibility is caused by E1A-induced cellular sensitivity to apoptosis or necrosis or to both cell death responses.

In the studies presented in this report, fibroblastic cells stably transfected with the E1A oncogene and expressing the E1A oncoprotein were used to test the relationship between E1A induction of cytolytic susceptibility and E1A sensitization to apoptotic injury. The results of

studies of the E1A expression level dependence of these two cellular phenotypes and of their linkage during *in vivo* selection and E1A mutational analysis suggest that the mechanism by which E1A induces cytolytic susceptibility involves E1A sensitization to killer cell-induced apoptosis.

MATERIALS AND METHODS

Cells and cell lines. NIH-3T3 cells were obtained from the American Type Culture Collection (ATCC). Several E1A-expressing cell lines derived from NIH-3T3 are represented in the figures as follows. The terms NIH-3T3+, 3T3-E1A and E1A 289R Hi (s. 1-3, 5 and Tables 1) refer to the cell line, 13-2, which expresses a high level of the 289R oncoprotein encoded by the E1A 13S cDNA [16]. E1A 289R Lo (Fig. 2A) refers to a similarly transfected clone, 13-3, which expresses a low level of E1A 289R [16]. E1A 243R Hi and E1A 243R Lo (Fig. 2A) refer to two clones, MT12SA and 12-3, which express either a high or low levels of E1A 12S cDNA-encoded protein, 243R, respectively, [16]. C3.11 (Fig. 2B) is a cell line established using the Lac Switch™ inducible mammalian expression system (Strategene, La Jolla, CA). For this purpose, the E1A gene was synthesized by polymerase chain reaction and replaced the CAT gene in the pOPRSVICAT plasmid. This plasmid was cotransfected with the p3'SS plasmid into NIH-3T3 cells, and transfected clones were selected in hygromycin and geneticin. The C3.11 subclone was selected for its expression of high level E1A upon induction with IPTG. 3T3-PSdl and 3T3-NCdl cells (Fig. 5) are NIH-3T3 clones established following transfection of NIH-3T3 cells with the E1A mutant genes, E1A-PSdl and E1A-NCdl, as described [9]. E1A-PSdl deletes all of E1A conserved region 1 (CR1) but does not affect expression of the N-terminal 22 amino acids of E1A [36]. The E1A-NCdl mutation deletes amino acids 61 through 85 but does not affect expression of either the E1A N-terminus or E1A CR1 [37]. The E1A-PSdl mutation eliminates the E1A gene regions required for oncoprotein binding to the cellular p300 transcriptional

coactivator and retinoblastoma (Rb)-family member proteins [3, 9, 36]. The E1A oncoprotein encoded by E1A-NCdl mutant gene retains the ability to bind p300 or Rb-family proteins [9, 56].

H4+ (Fig. 1) is an E1A-transfected H4 cell line, P2AHT2A [25]. The H4-derived cell line, H4-RG2-Clone 2, has been described [46]. This cell line was created by transfecting H4 cells with the E1A plasmid, 12S.RG2, which contains an E1A gene point mutation that results in an arginine-to-glycine change at the second amino acid in the amino-terminus of the E1A oncoprotein [57]. H4-RG2-Clone 2 expresses the mutant E1A protein at a high level [46]. The E1A-RG2 oncoprotein fails to bind cellular p300, but continues to bind Rb-family proteins [56].

RN12+ (Fig. 1; formal name RN12-1Agpt-A) is a cell line that expresses E1A at a high level and that was derived by transfection of RN12 cells with p1Agpt [24]. BHK21+ (Fig. 1) is an E1A expressing clone, D5, of BHK-21 cells [55].

Hamster embryo cells (HEC) were prepared and used as described [11]. ATL-1 and ATL-2 are cell lines derived from tumors developing in adult hamsters after serial *in vivo* transplantation of the Ad2-transformed HEC cell line, AdHE3 [14].

E1A expression levels in these cell lines were compared by immunoblotting using the monoclonal antibody, M73 [26]. High level E1A expression denotes an oncoprotein expression level that is comparable to that detected in virally transformed or virally infected rodent cells. Cells expressing E1A at low levels have been previously reported and compared to high level expressers [16].

All of these E1A-positive cell lines grow well *in vitro* and, of particular relevance for this study, do not spontaneously undergo apoptosis *in vitro*. Furthermore, where it has been tested (BHK-21-D5, Ad2HE3 and P2AHT2A), the E1A-positive cells are able to form tumors in immunodeficient animals [11, 19, 55], suggesting that these cells also do not spontaneously undergo apoptosis *in vivo*.

Assays of injury-induced apoptosis and necrosis. Each beauvericin, gramacidin and etoposide (VP16) lot (Sigma, St. Louis, MO) was titered against E1A-negative, NIH-3T3 cells and the E1A-positive, NIH-3T3 cell line, 13-2, across a broad range of concentrations to

determine optimal culture conditions for detecting E1A-specific sensitization to chemical injury. Initial studies of beauvericin-induced apoptosis were done using the apoptosis sensitive cell line, EL-4, as a control for comparison to E1A-positive cells. Assays of CL-induced apoptosis were done using the cytotoxic T lymphocyte (CTL) clone, 4.1, and the lectin, PHA-P, as described [18]. Apoptotic and necrotic cell death induced by these agents were confirmed by microscopic examination of nuclear morphological changes of injured cells [22] and were quantitated using 6-hr [^{51}Cr] release assays as described [55] and as validated in these studies. For cell morphology studies, cells stained with these DNA-binding dyes were scored based on the characteristics of aberrant chromatin organization as described [22]. The significance of the differences observed was estimated using Student's *t* test with JMP software from the SAS Institute. Low molecular weight DNA ("fragmented nuclear DNA") release from injured cells undergoing apoptosis [22] was used as a third means to quantitate apoptosis (Table 1).

RESULTS

E1A induction of cytolytic susceptibility correlates with sensitization to apoptotic injury. To begin to define the mechanism by which E1A sensitizes cells to killing by CL, we initially determined whether there was a correlation between E1A sensitization to apoptosis induced by both CL and chemically-induced injuries. The prediction was that different types of E1A-positive, cytolytic susceptible cells should simultaneously exhibit traits of CL-induced apoptosis and acquire sensitivity to apoptosis induced by unrelated proapoptotic, chemical stimuli. Mouse, rat, hamster and human cells that had been rendered highly susceptible to CL-induced lysis as a result of E1A transfection [16, 18, 45, 55] were tested for sensitivity to apoptosis induced by the potassium ionophore, beauvericin (Fig. 1). This proapoptotic agent was chosen because its mechanism of induction of apoptosis is distinct from the perforin/granzyme and Fas-ligand activities by which CL trigger apoptosis. Beauvericin induces release of intracellular calcium stores and appears to activate one or more cellular endonucleases that cause apoptosis in

susceptible cells [41]. Therefore, it was possible to ask the question of whether E1A expression induced sensitivity to diverse proapoptotic stimuli.

E1A-positive, CL-susceptible cells from all four species were highly sensitive to beauvericin-induced cell death, whereas the respective E1A-negative control cells were resistant (Fig. 1). To confirm that the cell death was apoptotic in nature, these E1A-positive cells were examined by fluorescence microscopy for beauvericin-induced cell shrinkage and nuclear chromatin condensation, which are diagnostic of apoptosis [22]. In all cases, E1A-positive cells showed apoptotic nuclear changes within 30 minutes after beauvericin treatment, whereas the cellular and nuclear morphologies of E1A-negative cells were unaffected by beauvericin. Beauvericin did not cause necrotic cell death of either E1A-positive or E1A-negative cells. Less than 5% of cells in all treated preparations showed the diffuse nuclear staining with ethidium bromide that is diagnostic of necrosis [22]. Therefore, the E1A-induced sensitization of cells to beauvericin was apoptosis-specific. Apoptosis-specific cell death was also observed with E1A-positive cells injured by CL. Fluorescence microscopy of E1A-positive NIH-3T3 cells that had been injured by cocultivation with the 4.1 clone of CL (at a 50:1 CL:target cell ratio) for 6 hours revealed apoptosis-specific nuclear fragmentation and condensation in the majority of target cells. Less than 5% of these CL-injured target cells in repeated assays showed cellular morphologies indicative of necrotic death (data not shown).

Another characteristic of cells undergoing apoptosis is degradation of their DNA into low molecular weight fragments as a result of internucleosomal chromatin cleavage [22]. This apoptotic response can be assessed qualitatively by DNA "laddering" patterns detected during agarose gel electrophoresis and quantitatively by measuring the percentage of DNA that is released as low molecular weight fragments [22]. DNA laddering was observed with beauvericin-treated, E1A-positive cells from all four species tested but not with DNA extracted from E1A-negative cells (not shown). DNA fragmentation was quantitated for E1A-positive and E1A-negative NIH-3T3 cells and compared with patterns of beauvericin-induced apoptotic nuclear morphology cell death, as quantitated by [^{51}Cr] release (Table 1). Both CL and

beauvericin caused a significant increase in cellular DNA fragmentation of E1A-positive, but not E1A-negative, cells. Beauvericin effects on E1A-positive cells were preceded by the nuclear morphology characteristic of apoptosis. The observations that E1A-positive cells from different species exhibited apoptotic, but not necrotic, cell death responses validated the subsequent used of the radiorelease assay to quantitate injury-induced apoptosis. This strong positive correlation between E1A-induced susceptibility to lysis by CL and E1A-induced sensitization to CL- and beauvericin-induced apoptosis suggested that target cell lysis and apoptosis are two manifestations of the same E1A activity.

E1A sensitization to apoptosis, like E1A-induced cytolytic susceptibility, depends on high level oncoprotein expression. Our previous studies showed that E1A-induced susceptibility to lysis by natural killer cells and activated macrophages is dependent on the relatively high levels of expression of E1A oncoproteins similar to those found during Ad infection of permissive cells or Ad-induced cell transformation [16, 19]. As a further test of the correlation between the requirements for E1A-induced cytolytic susceptibility and E1A sensitization to apoptotic injury, we used two different types of cells to evaluate the level of E1A oncoprotein expression required for beauvericin-induced apoptosis. NIH-3T3 cells stably transfected with cDNAs from either of the two major E1A mRNAs (13S or 12S) and clonally selected for expression of either high or low levels of the E1A oncoproteins (289R and 243R, respectively) were tested for sensitivity to beauvericin (Fig. 2A). Transfected cells expressing high levels of both E1A proteins were shown previously to be highly susceptible to lysis by natural killer lymphocytes (NK cells) and activated macrophages, whereas the transfectants expressing low levels of these E1A proteins remained resistant to lysis by both types of killer cells [16]. An identical pattern of sensitivity was detected to beauvericin-induced apoptosis. Cells expressing high level E1A 289R or 243R proteins were sensitive to beauvericin. In contrast, cells expressing low levels of E1A proteins were no more susceptible than E1A-negative NIH-3T3 cells (Fig. 2A).

We next tested whether clonal differences between these lines, other than E1A-induced effects, could explain the patterns of beauvericin sensitivity observed. For this purpose, a cell

line (C3.11) was created in which all cells in the population could be induced to express the E1A 289R oncoprotein when treated with IPTG. C3.11 cells expressed a barely detectable level of E1A 289R protein by Western blotting in the absence of IPTG induction (Fig. 2B). When treated with IPTG, essentially all cells in the population (>95% by E1A-specific immunofluorescence) expressed E1A 289R protein at a level as high as that detected in the E1A 289R-Hi expresser cell line represented in Fig. 2A. Uninduced C3.11 cells were resistant to lysis by different types of killer lymphocytes (not shown), indicating that the trace level of E1A expression in the uninduced state was insufficient to change their inherent cytolytic resistance. C3.11 cells became highly susceptible to CL-induced lysis during the first 24 hours after IPTG induction when the cells expressed high level E1A 289R protein (not shown).

This same pattern of dependence on E1A oncoprotein expression level was observed when a time-response study was done to assess sensitivity to chemically-induced apoptosis in C3.11 cells treated with beauvericin (Fig. 2B). In the uninduced state, cells expressing trace levels of E1A 289R were no more sensitive to beauvericin-induced apoptosis than E1A-negative NIH-3T3 cells (Fig. 2B). C3.11 cells became increasingly sensitive to injury-induced apoptosis through 36 hr of E1A induction (Fig. 2B, right three bars). Comparison of the E1A expression and apoptosis sensitization data revealed that there may also be a time dependence of E1A sensitization apoptosis. E1A expression level did not increase after 18 hr of IPTG stimulation (Fig. 2B). However, E1A-expressing cells exhibited increasing sensitivity to beauvericin-induced apoptosis throughout 36 hours of testing (Fig. 2B). Beauvericin-injured, E1A-positive C3.11 cells exhibited diagnostic apoptotic changes in nuclear morphology at each time point that were identical to those seen with cells stably expressing high level E1A 289R.

These data on C3.11 cells, coupled with those on cells stably expressing high or low levels of E1A shown in Fig. 2A, indicated that E1A sensitization to injury-induced apoptosis was dependent on a relatively high threshold level of E1A oncoprotein expression. The finding that both E1A-induced cytolytic susceptibility and E1A sensitization to chemically-induced apoptosis

were dependent on high level oncoprotein expression suggested that these two E1A-induced cellular phenotypes were manifestations of the same E1A activity.

E1A does not sensitize cells to injury-induced necrotic cell death. The DNA degradation patterns and nuclear morphology changes observed with beauvericin injured, E1A-positive cells were consistent with apoptosis. However, it has been reported with other types of cells that beauvericin can also cause cellular mitochondrial changes similar to those observed in cells undergoing necrotic cell death [41]. Furthermore, it has been suggested that CL can cause cellular necrosis, as well as apoptosis in some types of target cells [31], although most reported evidence indicates that apoptosis is the major form of CL-induced cell death [23]. To test whether any of the detected sensitization to cell death that is induced by E1A can be explained by increased cellular susceptibility to necrosis, E1A-positive and E1A-negative cells were compared for sensitivity to the potassium ionophore, gramacidin, an agent which causes necrotic cell death (R. Duke, personal communication). In these experiments, E1A-positive and E1A-negative NIH-3T3 cells were equally sensitive to gramacidin-induced (Fig. 3). The absence of an E1A sensitizing effect to necrotic cell death was also observed with matched E1A-positive and E1A-negative hamster, rat and human cells treated with gramacidin (not shown). Morphological studies of cells treated with gramacidin confirmed that the dying cells exhibited diffuse nuclear staining with ethidium bromide that is characteristic of necrotic cell death [22] and lacked the cell shrinkage and nuclear chromatin condensation seen with apoptotic cells. Therefore, high level E1A expression sensitizes cell to apoptosis induced by both CL and beauvericin, but E1A does not sensitize cells to gramacidin-induced necrotic cell death. These results are compatible with our reported observation that E1A-expressing cells are no more sensitive that E1A-negative cells to killing by antibody plus complement [18], another form of cellular injury that induces necrotic death.

Loss of E1A sensitization to injury-induced apoptosis is co-selected with loss of cytolytic susceptibility during neoplastic progression of Ad2-transformed rodent sarcoma cells. We have described an Ad2-transformed hamster sarcoma model in which weakly tumorigenic, E1A-

positive cells were adapted by serial *in vivo* passage to become highly tumorigenic in immunocompetent animals [14]. These "adapted" tumor cell lines, ATL-1 and ATL-2, retained identical patterns of adenoviral gene integration into the cellular genome and continued to express E1A oncoproteins at the same high levels seen with their weakly tumorigenic, parental sarcoma cell line, Ad2HE3. In comparison with parental cells, however, the ATL lines lost cytolytic susceptibility as evidenced by reduced killing by NK cells and tumor-cell-activated macrophages compared with Ad2HE3 cells [14].

To further test the correlation between E1A-induced cytolytic susceptibility and E1A sensitization to apoptotic injury, the two CL-resistant, ATL cell lines were compared for sensitivity to beauvericin-induced apoptosis with their CL-susceptible parental cell, Ad2HE3, and with the nontransformed hamster embryo (HEC) cells from which Ad2HE3 was derived (Fig. 4). The results showed that the CL-resistant ATL lines had also lost susceptibility to beauvericin-induced apoptosis relative to CL-susceptible Ad2HE3 cells and that the ATL lines were no more susceptible to beauvericin than CL-resistant, HEC cells. Therefore, these E1A-positive ATL cells which were selected *in vivo* for loss of cytolytic susceptibility were co-selected for loss of sensitivity to chemically-induced apoptosis. This linkage between the loss of these two cellular phenotypes strengthened the case for a causal relationship between E1A sensitization to apoptotic injury and E1A-induced cytolytic susceptibility.

E1A mutation that eliminates induction of cytolytic susceptibility also eliminates E1A-induced sensitization to apoptotic injury. We reported that first exon mutations involving either the N-terminus or conserved region 1 (CR1) of the E1A gene abrogated E1A-induced susceptibility of hamster cells to lysis by NK cells, despite continued high level oncoprotein expression following either viral infection or stable transfection [9]. These E1A gene regions are required for oncoprotein binding to the cellular transcriptional co-activator protein, p300. E1A first exon mutations that did not prevent E1A binding to p300 did not block E1A-induced cytolytic susceptibility. These results suggested the importance of E1A-p300 binding interactions for induction of cytolytic susceptibility.

E1A mutational analysis was used to test the correlation between E1A-induced sensitization to apoptotic injury and induction of susceptibility to lysis by killer cells. NIH-3T3 cells expressing high levels of two different mutant, E1A oncoproteins that differ in their binding to p300 were compared to cells expressing wild type E1A protein for sensitivity to apoptosis induced by killer cells and beauvericin (Fig. 5). 3T3-PSdl cells, which express an E1A mutant protein, E1A-PSdl, lacking the CR1 binding domain required for p300 binding [9] remained highly resistant to both killer cell- and beauvericin-induced apoptosis. 3T3-PSdl cells were as resistant to by injuries as nontransfected NIH-3T3 cells. In contrast, 3T3-NCdl cells, which express an E1A mutant protein, E1A-NCdl, that continues to bind p300 as well as wild type E1A protein [9] were as sensitive to both proapoptotic injuries as target cells expressing wild type E1A. Cells expressing either wild type E1A or E1A-NCdl proteins exhibited nuclear morphologies and low molecular weight DNA fragment release patterns diagnostic of apoptosis, whereas cells expressing E1A-PSdl protein were indistinguishable from E1A-negative cells in these assays. The observation that the E1A-PSdl mutation abrogated E1A-induced cellular sensitization to apoptotic injury suggested the importance of E1A-p300 interactions in this E1A activity. However, since the PSdl mutation can also affect E1A binding to Rb-family proteins, these data did not exclude a role for E1A-Rb interactions in sensitization to injury-induced apoptosis.

To refine the analysis of the requirement for E1A-p300 binding interactions for cellular sensitization to proapoptotic injuries, human H4 fibrosarcoma cells expressing either wild type (wt) E1A oncoprotein or an E1A mutant protein encoded by E1A 12S.RG2, were compared for susceptibility to injury-induced apoptosis (Fig. 6). The E1A mutant gene, 12S.RG2, contains a point mutation that causes an arginine-to-glycine switch at the second amino acid of the E1A oncoprotein and results in loss of p300 binding [57] but does not affect Rb-family protein binding by E1A (our unpublished data). The H4 cell line, RG2-Clone 2, was selected for these experiments because it expresses high level of the mutant E1A RG2 protein. Three types of proapoptotic injuries — human NK cells (Hu NK), beauvericin (BR) and etoposide (Etop) —

were used to test the effect of the RG2 mutation on the ability of E1A to sensitize human cells to apoptosis. Etoposide is a topoisomerase II inhibitor that triggers apoptosis in susceptible cells [32]. Fibrosarcoma cells expressing wild type (wt) E1A were susceptible to apoptosis induced by all three injuries (Fig. 5B). In contrast, H4-RG2-Clone 2 cells were as resistant to all three injuries as parental, E1A-negative fibrosarcoma cells. Subsequent coimmunoprecipitation studies confirmed that the E1A-RG2 mutant protein expressed in these cells failed to form complexes with the p300 transcriptional coactivator but did form complexes with Rb-family member proteins (data not shown). These data confirm the observations using the larger E1A deletion mutation, PSdl, by showing that an E1A mutation that eliminates oncoprotein binding to p300 also eliminates the E1A activity that sensitizes cells to injury-induced apoptosis and suggest that E1A binding to cellular Rb-family proteins is insufficient to sensitize cells to apoptotic injury.

DISCUSSION

These studies provide several types of evidence that indicate a cause and effect relationship between E1A sensitization to injury-induced apoptosis and E1A induction of cellular susceptibility to lysis by killer cells. First, the association between these two E1A-induced cellular phenotypes was found to be a general property of cells from four different species (Fig. 1). Second, there were similar dose-response relationships between E1A oncoprotein expression and sensitization to injury-induced apoptosis (Fig. 2) and E1A oncoprotein expression and induction of cytolytic susceptibility [16, 19]. In both cases, high level expression similar to that observed during viral infection or virus-induced neoplastic transformation of cells is required for the E1A-related cellular phenotype. In contrast, other E1A activities, including transcriptional activation [59], transcriptional repression [53], cellular immortalization [1] and support of adenoviral replication [27] require only low levels of E1A protein expression. Third, cell selection *in vivo* for loss of E1A-induced cytolytic susceptibility co-selects for loss of E1A sensitization to injury-induced apoptosis (Fig. 4). Fourth, E1A gene mutations that caused the

oncoprotein the lose the ability to induce cytolytic susceptibility also caused loss of E1A-induced sensitization to injury-induced (Figs. 5). Fifth, E1A sensitization to injury-induced cell death was specific for apoptosis, since E1A did not sensitize cells to injury-induced necrosis (Fig. 3). This specificity of E1A sensitization for apoptotic injury is compatible with our reported observation that E1A does not sensitize cells to necrotic cell death induced by treatment with antibody plus complement [18]. All of these observations support the conclusion that E1A sensitization to apoptotic injury and E1A-induced susceptibility of target cells to lysis by killer cells are manifestations of the same E1A oncoprotein activity.

CL can induce both apoptotic and necrotic cell death responses, depending on the target cell tested [20, 28, 31, 54, 60]. The data in this report show that the major mechanism by which E1A renders cells more susceptible to CL-induced lysis is E1A sensitization to apoptosis, not necrosis. E1A expression did not sensitize cells to either CL-induced or chemically (gramacidin)-induced necrosis. These results suggest the conclusion that pronecrotic activities of CL are not important in the *in vivo* rejection of E1A-expressing tumor cells by immunocompetent animals.

There was a strong correlation between increased levels of E1A oncoprotein expression and sensitization to killer cell-induced apoptosis in these studies (Fig. 2). These results provide an explanation for the E1A-expression-level dependence of expresser cell susceptibility to killing by NK cells, activated macrophages and tumor necrosis factor (TNF) alpha. For example, we reported that adenovirus-induced susceptibility of infected hamster fibroblasts to killing by activated macrophages increases with increasing multiplicity of viral infection and the concomitant increased expression of viral early proteins, including E1A [12]. We also observed that high, but not low, level E1A oncoprotein expression causes increased susceptibility of BHK-21 cells to NK cell killing [19] and of NIH-3T3 cells to killing by NK cells, activated macrophages and recombinant TNF alpha [16]. All three of these types of immune-mediated injuries can induce apoptosis in susceptible target cells. These correlations lead us to suggest that the loss of tumorigenicity of E1A-expressing BHK-21 cells in immunocompetent (but not

immunodeficient) animals represents the *in vivo* consequence of E1A sensitization of tumor cells to immune-mediated apoptosis [19, 55].

The reasons for the time delay between reaching maximum E1A expression in the inducible NIH-3T3 cell line, C3.11, and detection of maximum sensitivity to apoptotic injury (Fig. 2B) are unknown. It is possible that, as the E1A oncoprotein is expressed, it must alter the function of one or more targeted cellular molecules, such as the p300 transcriptional coactivator, before complete sensitization to apoptotic injury can be achieved. This would suggest that a certain threshold of titration of the targeted molecule(s) by E1A must occur before cellular sensitization to apoptotic injury is complete. We have reported a correlation between E1A interactions with the p300 transcriptional coactivator and induction of susceptibility of Ad-infected and E1A-transfected cells to killing by natural killer lymphocytes [9]. Most reports indicate that E1A-p300 interactions result in repression of p300-dependent cellular transcription. It is possible, therefore, that E1A must titrate a p300-dependent transcriptional response to sensitize cells to injury-induced apoptosis.

There is evidence that E1A itself can also initiate apoptosis, either directly or indirectly, without prior cellular injury. When E1A is expressed during either viral infection or initiation of oncogene-induced cellular immortalization, it triggers apoptosis without a requirement for any subsequent cellular injury [4, 6, 21, 29, 30, 38-40, 42, 43, 47, 48, 58]. Most of these reports suggest that this direct induction of cellular apoptosis by E1A is the consequence of its stimulation of unscheduled cellular DNA synthesis that, in turn, triggers a p53-dependent apoptosis pathway. In studies from one laboratory, it was shown that E1A can promote apoptosis in virally infected cells indirectly through transcriptional activation of the adenoviral E4 gene which then triggers p53-independent apoptosis [35, 52]. In another report, E1A was shown to cause apoptosis of virally infected cells independently of both E4 and p53 expression [7].

The requirement for injury of cells stably expressing E1A to undergo apoptosis distinguishes the E1A activity described in this report from studies in which E1A expression itself causes cellular apoptosis during viral infection. The E1A-transfected cell lines studied here, and others we have

tested, are viable during long-term tissue culture passage and, where evaluated, have induced tumors in immunodeficient animals [8, 9, 13, 55]. Therefore, in these selected, E1A-transfected cell lines, there are not apparent adverse effects of E1A oncoprotein expression until the cells are exposed to proapoptotic injuries. The viability of these E1A-expressing cells does not require co-expression of other collaborating genes, such as E1B [43].

Other reports of E1A-induced sensitization of cells to nonimmune, proapoptotic injuries have demonstrated a requirement for p53 expression for this E1A effect [33, 34]. This p53 requirement is also similar to that reported in most of the aforementioned studies of E1A-related apoptosis occurring during viral infection or the establishment of the immortalized state. Our unpublished results indicate that E1A-induced cytolytic susceptibility and E1A sensitization to immune-mediated apoptosis are independent of p53 expression (Cook, JL, Routes, BA, Walker, TW and Colvin, KL, submitted for publication). Whether all of these relationships between cellular sensitization to apoptotic injury and E1A expression during infection, immortalization and stable transfection using different assays of apoptosis are different manifestations of the same E1A activity or whether there are different E1A-controlled cellular pathways that affect the cellular apoptotic response are questions that will require further, direct comparison studies.

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FIGURE LEGENDS

Figure 1. Increased beauvericin sensitivity of NK-susceptible E1A-expressing cell lines from four different species. E1A-positive (dark bars) and E1A-negative mouse, rat, hamster and human fibroblastic cells were tested for sensitivity to beauvericin-induced killing in 6 hour [^{51}Cr] [^{51}Cr]-release assays. Bars represent the mean ± SEM results of three (RN12, BHK-21 and H4) to eight (NIH-3T3) experiments using beauvericin at a final concentration of 10 uM (mouse, rat and human cells) or 13 uM (hamster cells). E1A-positive cells of all four types were significantly more sensitive to beauvericin-induced cell death than the respective E1A-negative cells ($p < 0.05$).

Figure 2. E1A expression-level-dependence of cellular sensitivity to apoptotic injury. NIH-3T3 cells expressing E1A proteins at high or low levels (as indicated) following either **(A)** stable transfection with E1A 13S (289R protein) or 12S (243R protein) cDNAs, respectively [16] or **(B)** IPTG induction of E1A 13S cDNA (289R protein) expression (C3.11 cells) were tested for sensitivity to apoptosis induced by beauvericin at a final concentration of 13 uM. Bars in Fig. 2A represent the mean ± SEM results of three to six experiments. Cells expressing high levels of either E1A 289R or E1A 243R were significantly more sensitive to beauvericin-induced apoptosis than either E1A-negative, NIH-3T3 cells or cells expressing the respective E1A proteins at low levels ($p < 0.05$). Bars in Fig. 2B represent the mean ± SEM results of four assays in which the beauvericin sensitivities of C3.11 cells were tested before and at the indicated times after IPTG induction of E1A 289R expression. E1A-negative NIH-3T3 cells and the stably transfected, E1A-positive cell line, 13-2, were used as beauvericin-resistant and beauvericin-sensitive controls, respectively. IPTG-induced C3.11 cells were significantly more sensitive to beauvericin-induced apoptosis than untreated control cells at 24 and 36 hours after IPTG treatment ($p < 0.05$ by ANOVA using Dunnett's comparison).

Figure 3. Lack of E1A sensitization of NIH-3T3 cells to necrotic cell death. E1A-negative and E1A-positive (13-2) cells were compared for sensitivity to chemically-induced necrosis at the indicated concentrations of gramacidin. Points represent the mean ± SEM results of three experiments. E1A-positive cells were not more sensitive to gramadicin-induced necrosis than E1A-negative cells ($p > 0.10$).

Figure 4. Loss of apoptosis sensitivity of E1A-positive fibrosarcoma cells following *in vivo* selection for loss of susceptibility to NK killing during serial tumor transplantation. Tumor lines, ATL-1 and ATL-2, derived from *in vivo* passage of the NK-susceptible hamster cell line, Ad2HE3, were compared to parental cells for susceptibility to apoptosis induced by beauvericin at a final concentration of 10 uM. Primary HEC (from which Ad2HE3 were derived) were used as apoptosis-resistant control cells. Ad2HE3 cells are highly susceptible to lysis by hamster NK cells, whereas ATL-1, ATL-2 and HEC cells are NK resistant [14]. Bars represent the mean ± SEM results of 5 experiments. ATL-1, ATL-2 and HEC cells were significantly less susceptible to beauvericin-induced apoptosis than Ad2HE3 cells ($p < 0.05$).

Figure 5. Failure of E1A mutant oncoproteins that cannot bind the cellular p300 transcriptional co-activator to sensitize mouse **(A)** and human **(B)** cells to apoptotic injuries. **(A)** NIH-3T3 transfected clones, 3T3-PSdl and 3T3-NCdl, were compared with E1A-negative (NIH-3T3) and E1A-positive (3T3-E1A) controls for sensitivity to apoptosis induced by either cytotoxic lymphocytes (CL; 4.1 cells at a killer cell to target cell ratio = 6:1) or beauvericin (final concentration = 10 uM). Bars represent the mean ± SEM results of three experiments with each type of injury. Binding of wild type or mutant E1A proteins to p300 as detected by immunoprecipitation with E1A-specific antibody followed by immunoblotting with anti-p300 antibody as described [9] is indicated. 3T3-PSdl cells (no E1A-p300 binding detected) were significantly less susceptible to apoptosis induced by both types of injuries than cells expressing either wild type E1A or E1A-NCdl ($p < 0.05$), both of which showed E1A-p300 binding. In

contrast, 3T3-NCdl cells were equally susceptible to CL and beauvericin compared with 3T3-E1A cells. **(B)** Human H4 fibrosarcoma cells transfected with the E1A point mutation gene, 12S.RG2, were compared with nontransfected parental cells and cells transfected with the wild type (wt) E1A gene for susceptibility to three types of proapoptotic injuries. Hu NK = human natural killer cell assay results, using a 200:1 blood mononuclear cell to target cell ratio and an cocultivation period of 6 hours. BR = beauvericin at final concentration of 11 uM in 6 hour apoptosis assays. Etop = etoposide at a final concentration of 100 ug/ml in 18 hour apoptosis assays. Bars represent the mean ± SEM results of two (BR) or three (Hu NK and Etop) experiments with each type of injury. H4-RG2-CL39 cells were significantly less susceptible to injury-induced apoptosis triggered by all three types of injury than cells expressing E1A wt ($p < 0.05$), but RG2-CL-39 cells were not significantly more susceptible to proapoptotic injury than parental, E1A-negative H4 cells.

TABLE 1

Evidence for apoptotic death of E1A-positive NIH-3T3 cells injured by cytolytic lymphocytes or beauvericin

Injury	Target Cell	
	NIH-3T3	3T3-E1A
	(percentage)	
Cytolytic Lymphocytes		
Fragmented nuclear DNA (2 hr)	33 ± 2	70 ± 3
[^{51}Cr] release (6 hr)	19 ± 5	58 ± 5
Beauvericin		
Apoptotic nuclei (30 min)	10 ± 2	85 ± 5
Fragmented nuclear DNA (2 hr)	13 ± 5	47 ± 8
[^{51}Cr] release (6 hr)	16 ± 2	64 ± 3

Note. Enumeration of apoptotic cells and quantitation of fragmented nuclear DNA release were done as described (Duke & Cohen, 1992). Quantitation of cell death was estimated by radiolabel release as described (Walker *et al.*, 1991). Each set of comparison data represents the mean ± SEM results of at least three experiments. For each index of measurement, the E1A-positive cells (3T3-E1A) were significantly more senstive to apoptotic injury than the E1A-negative cells (NIH-3T3) ($p < 0.05$).

Fig. 1

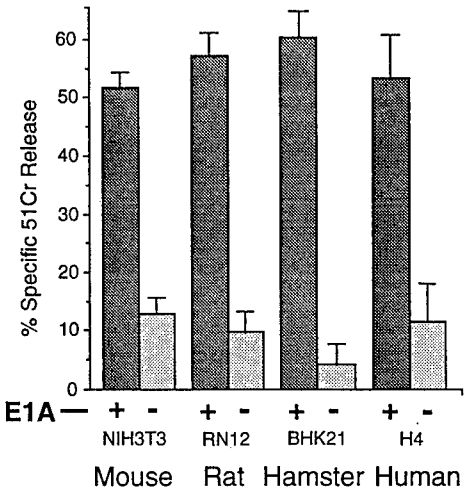


Fig. 3

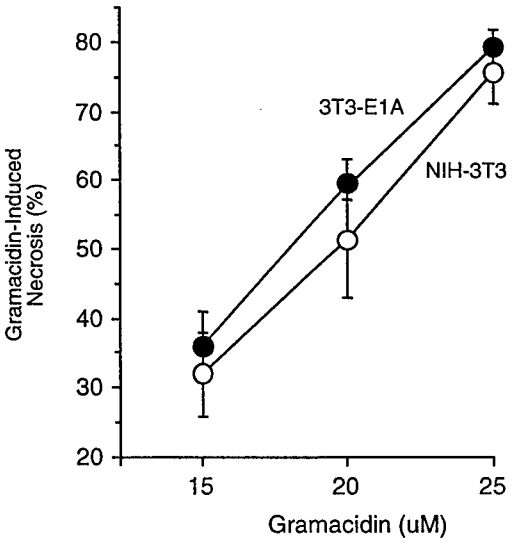


Fig. 2

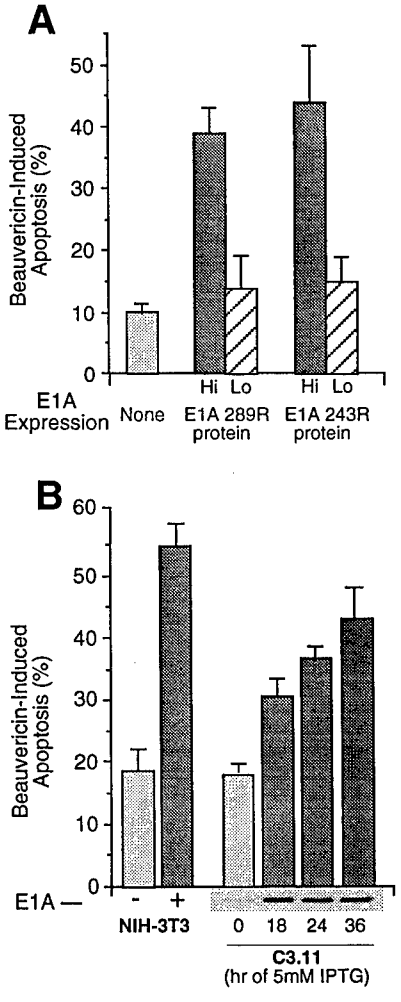


Fig. 4

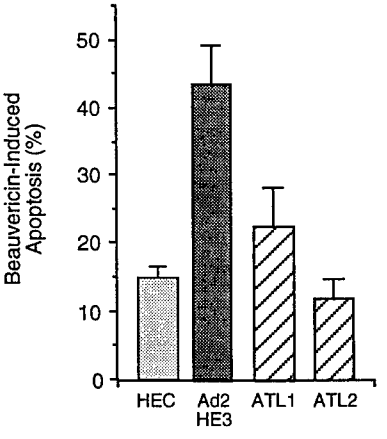
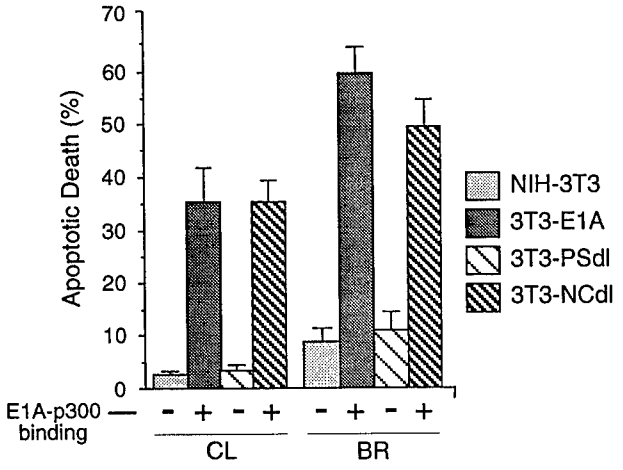
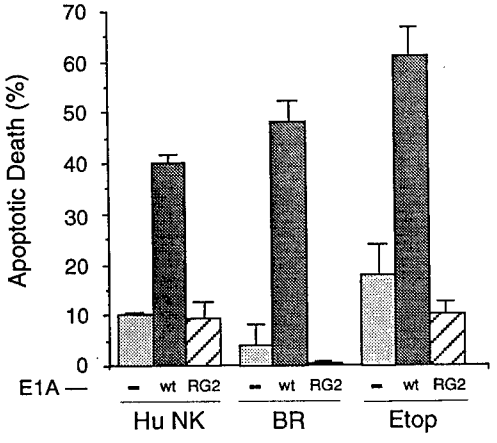


Fig. 5

A


Apoptotic Death (%)
70
60
50
40
30
20
10
0
NIH-3T3
3T3-E1A
3T3-PSdl
3T3-NCdl
E1A-p300 binding
- + - +
- + - +
CL
BR

B


Apoptotic Death (%)
70
60
50
40
30
20
10
0
E1A
- wt RG2
- wt RG2
- wt RG2
Hu NK
BR
Etop

Running title: E1A sensitization to apoptotic injury

E1A Oncogene-Induced Cellular Sensitization to Immune-Mediated Apoptosis is Independent of p53 and Resistant to Blockade by E1B 19 kD Protein

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ABSTRACT

E1A oncogene expression sensitizes mammalian cells to apoptosis triggered by cytolytic lymphocytes (CL) [16]. Most studies suggest that E1A-induced apoptosis involves a p53-dependent cellular pathway that is blocked by the E1B 19 kD gene product. In this study, the roles of p53 and E1B 19 kD were tested for E1A sensitization to CL-induced apoptosis in contrast with apoptosis triggered by TNF alpha or chemical injuries. E1A sensitization to immune-mediated (CL- or TNF-induced) apoptosis was independent of p53 expression and was resistant to blockade by E1B 19 kD protein in mouse and hamster cells. In contrast, the p53 requirement for chemically induced apoptosis of E1A-sensitized cells varied with the agent used to treat cells. Apoptosis induced by diverse chemical agents (hygromycin, beauvericin, etoposide, H_2O_2) was blocked by E1B 19 kD expression. Therefore, both the p53-dependence and E1B 19 kD blockade of E1A-induced cellular sensitization to apoptotic injury can depend on the type of proapoptotic injury tested. These data suggest that the mechanisms by which E1A sensitizes tumor cells to immune-mediated apoptosis and to rejection by immunocompetent animals does not require cellular expression of wild type p53 and can function independently of the Bcl-2-like, antiapoptotic mechanisms of E1B 19 kD.

Key words: adenovirus, E1A, cytolytic lymphocyte, tumor necrosis factor alpha, apoptosis, p53, E1B 19kD.

INTRODUCTION

Expression of the E1A oncogene of human adenovirus (Ad) types 2 and 5 renders mammalian cells from several species susceptible to lysis by components of the host cellular immune response, including both major types of cytolytic lymphocytes (CL) — natural killer (NK) cells and cytotoxic T lymphocytes (CTL) — activated macrophages, and tumor necrosis factor alpha (TNF) [1, 8, 23, 25, 32, 44, 71, 80]. We and others have postulated that this E1A-induced cytolytic susceptibility explains the inability of Ad-transformed cells and E1A-expressing tumor cells to form tumors in immunocompetent animals [14, 17, 68, 71, 75, 79, 90]. This hypothesis is supported by our recent observation that elimination of innate and specific cellular immunity in vivo also progressively eliminates host rejection of E1A-positive sarcoma cells (Routes, J.M., Ryan, S., Li, H., Steinke, J. and Cook, J.L., submitted for publication). The molecular mechanisms by which E1A sensitizes cells to these immune-mediated injuries are unknown, however.

We have reported that E1A sensitizes cells to killing by both types of CL-induced cellular injuries — degranulation-dependent and Fas-dependent injuries [24]. Fas-dependent injury by CL is specific for apoptosis [reviewed in 2]. CL-induced, degranulation-dependent injury mediated by the collaborative interaction of perforin and granzymes can cause either apoptosis or necrotic cell death, apparently depending on the target cell type and assay conditions [27, 82, 89]. Our recent studies indicate that degranulation-dependent injury by CL causes apoptotic, and not necrotic, cell death in E1A-expressing rodent fibroblasts [16]. We were, therefore, interested in testing the cellular requirements for CL-induced apoptosis of E1A-positive cells.

p53 antioncogene expression is required in other circumstances in which E1A induces apoptosis, including viral infection and oncogene-induced cellular immortalization [9, 29, 53, 62, 66, 67, 73, 93]. Testing of the role of p53 in E1A-induced sensitivity to CL-induced apoptosis has not been reported. However, the following indirect evidence suggested that p53 could be involved. It has been proposed that E1A-induced, p53-dependent apoptosis caused by other stimuli requires E1A binding to the p300 protein [66], which is a transcriptional co-activator for

p53 [52, 78, 83]. Furthermore, E1A-induced cellular sensitization to killing by CL maps to the p300 binding regions of E1A [15].

E1A-related, p53-dependent apoptosis can be blocked by co-expression of the Ad E1B 19 kD oncogene [4, 29, 67, 72]. E1B might protect virally infected cells from undergoing premature, E1A-induced apoptosis. The E1B protective effect might also explain the increased efficiency of cellular immortalization of rodent cells cotransfected with E1A and E1B compared to cells transfected with E1A alone. E1B 19 kD blockade of apoptosis triggered by exogenous cellular injuries is variable. E1B 19 kD can block TNF-induced apoptosis [10, 37, 42, 93], but this can be cell-type- or species-specific [48, 87]. This is similar to the cell-system-specific, TNF blocking effect of Bcl-2 [69], the antiapoptotic cellular gene with which E1B shares limited sequence homology and functional activity. E1B 19 kD and Bcl-2 blockade of Fas-induced apoptosis is also variable [10, 12, 60, 77, 84]. Our previous reports showed no E1B blockade of E1A-induced susceptibility to CL-induced killing of Ad-infected, Ad-transformed or oncogene-transfected tumor cells [25, 71, 90], suggesting that E1B 19 kD might not effectively block E1A-induced sensitization to CL-induced apoptosis.

In these studies, p53-negative and p53-positive mouse cells were compared for E1A-induced sensitization to immune-mediated or chemically triggered apoptosis. Mouse and hamster cells expressing E1A alone or co-expressing E1A plus E1B 19 kD were contrasted to test the efficacy of E1B blockade of E1A sensitization to apoptosis. The results showed that expression of wild type p53 was not required for E1A to sensitize cells to immune-mediated apoptosis, but that some types of chemically induced injuries required p53 to trigger apoptosis of E1A-sensitized cells. E1A sensitization to immune-mediated apoptosis was resistant to E1B 19 kD blockade, whereas E1B blocked apoptosis induced by several different types of chemical injuries. The implications of these observations for the mechanisms by which E1A sensitizes cells to immune-mediated apoptosis and to rejection by immunocompetent animals are discussed.

MATERIALS AND METHODS

Cell lines

NIH-3T3 cells were obtained from the ATCC. The E1A-positive clone, 13-2 (represented in the Figures as 3T3-E1A), has been described [23]. 13-2 expresses high level E1A 289R oncoprotein encoded by the E1A 13S cDNA. (10)3 cells are 3T3-like, mouse fibroblasts with a mutation that creates a stop codon in the p53 coding sequence, and have no detectable p53 protein [41]. (10)3-E1A (Figs. 3, 6 and 7 and Table 1), refers to a (10)3 subclone, A22, that expresses E1A at a comparable level to 13-2, by immunoblotting with the monoclonal antibody, M73 [40]. A22 was created by transfection of (10)3 with the plasmid, p1A-pac, and selection in puromycin. The E1A-positive, Saos-2 human osteosarcoma cell line was provided by S. Frisch and expresses E1A 243R protein (encoded by the E1A 12S cDNA) at a level comparable to the E1A in 13-2 cells.

Three cell lines were used for studies testing E1B 19 kD effects on sensitivity to apoptotic injury (Fig. 4) — NIH-3T3 (E1A and E1B negative), F411 (E1A-positive, E1B-negative) and E1PB9 (E1A-positive, E1B 19 kD-positive). F411 was created by transfecting NIH-3T3 with p1A-pac and selecting E1A-expressing clones in puromycin. E1PB9 was created by cotransfecting NIH-3T3 with the plasmids p5XhoI-C [3] and pPUR (Clontech, Palo Alto, CA) and selecting E1A-expressing clones in puromycin. A second selection was done to identify E1A-positive clones that expressed E1A 19 kD protein, as assessed by immunoblotting using the antibody, 1G11 (Oncogene Sciences, San Diego, CA). The BHK-21 lines (Fig. 7C) have been described [26, 90]. The cell line represented as BHK-E1A+E1B is BHK-D5 that expresses E1A and E1B 19 kD oncoproteins at high levels. The cell line represented as BHK-E1A is BHK-B2 that expresses only E1A oncoproteins at the same high level as BHK-D5 cells [26].

Assays of injury-induced apoptosis

CL-induced apoptosis of mouse target cells was assessed using the mouse CTL clone, 4.1 [46], in lectin-dependent cellular cytotoxicity assays as described [24]. 4.1 kills by degranulation-

dependent mechanisms in calcium-rich medium and cannot mediate Fas-dependent killing without prior activation of Fas-ligand expression [24]. Degranulation-dependent, Fas-independent killing by 4.1 was used here. CL-induced apoptosis of human osteosarcoma cells was assessed using human NK cells as described [25, 71]. Recombinant mouse TNF alpha (Genzyme, Cambridge, MA), hygromycin (Cal Biochem, La Jolla, CA), beauvericin (Sigma, St Louis, MO), etoposide (Sigma, St. Louis, MO) and H_20_2 (Bergen Brunswig, Orange , CA) were tested in dose-response studies of NIH-3T3 and E1A-positive, 13-2 to define optimal concentrations for detecting E1A-specific sensitization to apoptotic injury (Table 1). Apoptosis induced by these agents was assessed by evaluating the nuclear morphology of injured cells [33]. All of these injuries induced nuclear fragmentation and chromatin condensation in E1A-positive target cells. In contrast, less that 5% of cells injured with any agent showed the diffuse nuclear staining with ethidium bromide that indicates cellular necrosis. Low molecular weight DNA release from injured cells [33] was used as a second test to define apoptotic cell death. Our studies have validated the use of [^{51}Cr] release (6-hr for CL, NK, and beauvericin and 18 hr for TNF, hygromycin, etoposide, and H_20_2) to quantitate apoptotic death in assays such as these where injury-induced necrosis has been excluded [16]. The significance of the differences in apoptotic cell death was estimated by analysis of variance using the JMP program from SAS Institute.

RESULTS

E1A-induced sensitization of target cells to immune-mediated apoptosis does not require p53 expression.

To test the p53 requirement for immune-mediated apoptosis, assays of degranulation-dependent killing by CL were used to compare apoptotic injury of E1A-expressing, p53-negative and p53-positive mouse fibroblasts. E1A expression caused p53-negative, (10)3 cells to become as sensitive to CL-induced apoptosis as E1A-positive, p53-positive, NIH-3T3 cells, across a tenfold range of CL to target cell ratios (Fig. 1). Therefore, p53 expression was not required for either

the mechanism by which CL injured cells or the mechanism by which E1A sensitized cells to apoptotic injury. Identical results were obtained with the p53-negative, human Saos-2 cells [31], in which E1A expression caused increased susceptibility to apoptosis induced by human NK cells (Fig. 2).

In addition to cytotoxic T lymphocytes and NK cells (the main types of CL in vivo), the activated macrophage is another major component of the host cellular immune response to neoplastic cells. The main cytotoxic mediator used by activated macrophages is the cytokine, TNF alpha, which is responsible for the majority of macrophage cytolytic activity against E1A-positive cells [23]. TNF can induce p53-dependent cellular responses [43, 94], but can also trigger apoptosis in the absence of p53 [47, 95]. To test the p53 requirement for E1A sensitization to TNF-induced apoptosis, the same set of p53-negative and p53-positive mouse cells used for CL apoptosis assays was tested for apoptotic sensitivity in TNF dose-response experiments (Fig. 3). E1A expression sensitized p53-negative and p53-positive mouse fibroblasts equally to TNF-induced apoptosis. Therefore, p53 expression was not required for the E1A activity that sensitizes cells to injury by either CL (Figs. 1 and 2) or TNF (Fig. 3).

The p53 requirement for E1A-induced cellular sensitization to apoptosis is dependent on the proapoptotic injury tested.

In contrast to these results of immune-mediated cellular injury, it is reported that E1A sensitization of mouse fibroblasts to chemotherapeutic agents and ionizing irradiation is strictly p53-dependent [55]. This suggested the possibility that the apoptotic sensitivity of E1A-positive cells could be a function of a p53 requirement for *triggering* apoptosis by certain injuries, and not a reflection of whether E1A had *sensitized* the cells to injury-induced apoptosis *per se*. To test this, different chemical injuries (hygromycin, beauvericin, etoposide and H_2O_2) were contrasted with CL- and TNF-induced injuries for induction of apoptosis in p53-positive and p53-negative mouse cells expressing E1A (Table 1). E1A-positive, p53-positive cells were susceptible to apoptosis induced by all six types of injuries. In contrast, E1A-positive, p53-negative cells were susceptible to apoptosis induced by CL, TNF and hygromycin, but were

highly resistant to apoptosis induced by beauvericin, etoposide and H_2O_2. The hygromycin result showed that some chemical injuries do not require p53 expression to trigger apoptosis in E1A-sensitized cells. The beauvericin, etoposide and H_2O_2 results showed that other chemical injuries can require p53 expression to trigger apoptosis in E1A-sensitized cells. More importantly as a general observation was that the apparent sensitivity of E1A-expressing cells to apoptosis is completely dependent on the type of proapoptotic injury chosen for the experiment.

E1B 19 kD protein cannot prevent E1A sensitization to immune-mediated apoptosis but can block apoptosis induced by chemical injuries in NIH-3T3 cells.

E1B 19 kD is an adenoviral gene-encoded protein that is a member of a family of viral and cellular proteins, which inhibit apoptosis, with Bcl-2 being the prototype [reviewed in 92]. E1B 19 kD, like Bcl-2, can block some types of proapoptotic injuries, but not others [12, 60, 64, 69]. We reported that E1B expression does not prevent E1A induction of cellular susceptibility of rodent or human cells to lysis by NK cells, CTL or activated macrophages [19, 22, 24-26, 50, 71, 90].

Studies were done to test the prediction from those observations that E1B 19 kD would not block CL- or TNF-induced apoptosis of E1A-positive cells and to contrast the E1B effects on immune-mediated apoptosis with its effects against chemically triggered apoptosis of E1A-positive cells. p53-positive, NIH-3T3 cell clones were used that expressed either E1A alone or both E1A and E1B 19 kD genes. The levels of E1A and E1B 19 kD oncoprotein expression in these clones as detected by immunoblot with oncoprotein-specific monoclonal antibodies were comparable to the oncoprotein levels commonly detected in oncogene-transformed mouse, rat, hamster and human cell lines (data not shown). The apoptotic sensitivities of these cells were contrasted using six injuries – CL, TNF, hygromycin, beauvericin, etoposide and H_2O_2 – under conditions that had been optimized for causing apoptosis of cells expressing only E1A (Fig. 4). E1B 19 kD had no significant inhibitory effect on apoptosis induced by either CL or TNF in these assays. In contrast, E1B 19 kD co-expression blocked E1A-induced sensitization to

apoptosis induced by hygromycin, beauvericin, etoposide and H_2O_2 down to levels that were not significantly different from those observed with E1A-negative, NIH-3T3 cells. The ability of E1B 19 kD to block E1A sensitization to apoptosis was not linked to the p53-dependence of the injury (Table 1), since hygromycin (a p53-independent injury) and all three p53-dependent injuries (beauvericin, etoposide and H_2O_2) were blocked, but CL and TNF (both p53-independent injuries) were not.

E1B 19 kD protein expression does not block E1A sensitization immune-mediated apoptosis in either mouse fibroblasts or hamster sarcoma cells.

The ability to detect blockade of injury-induced apoptosis by E1B 19 kD and the functionally similar antiapoptotic molecule, Bcl-2, has varied depending on the injury and cell system tested [10, 37, 42, 48, 69, 87, 93]. The data in Figure 4 showed that, using relatively high effector to target cell ratios (i.e., 50 CL to 1 target cell) at which CL-induced apoptosis of E1A-sensitized cells is maximized, E1B 19 kD co-expression did not block E1A sensitization to CL-induced apoptosis in a single NIH-3T3 cell line.

Several possibilities were considered to explain this failure of apoptosis blockade by E1B, including inadequate E1B 19 kD protein expression, another property unique to that cell line (E1PB9) which could prevent E1B 19 kD from blocking apoptosis, and excessive CL-induced cell injury which prevented detection of limited E1B 19 kD blockade. E1B 19 kD protein expression level in E1PB9 cells was compared quantitatively to that detected in the E1A+E1B expressing human cell line, 293 [39], which, in our comparison immunoblotting studies, consistently expresses E1B 19 kD at the highest level among several adenovirus-transformed or oncogene-transfected mammalian cell lines. E1PB9 expressed a steady state level of E1B 19 kD protein that was comparable to that of 293 cells (Fig. 5). Therefore, low level expression of E1B 19 kD protein in E1PB9 did not explain the failure of E1B to block CL-induced apoptosis of these E1A-sensitized cells.

Experiments were done to test the possibilities that the failure of E1B 19 kD blockade was caused by another trait (unrelated to E1B protein expression level) unique to the E1PB9 or to

NIH-3T3 cells. We also tested the possibility that the high killer cell to target cell ratio used in the studies shown in Table 1 created a proapoptotic injury that could not be overcome by E1B 19 kD. For these purposes, we tested different target cell types and a range of CL-to-target-cell ratios. Mouse (10)3 fibroblasts expressing either E1A alone or E1A+E1B 19 kD were compared with similar NIH-3T3 clones (Fig. 6). Even at low CL-to-target-cell ratios, there was no significant, E1B-related reduction in the apoptotic sensitivity of either type of E1A-positive mouse fibroblast (Figs. 6A and 6B). In fact there was a trend toward increased CL-induced apoptosis of the E1A+E1B positive (10)3 cells vs. the E1A-only expressing (10)3 cells across the whole range of E:T ratios (Fig. 6B). These findings were consistent with our reports that E1A-sensitized, BHK-21 hamster cells are no less sensitive to CL-induced killing when they co-express high levels of E1B 19 kD [26] and that human 293 and HE1-E1 cells, both of which co-express E1A and high levels of E1B 19 kD, are highly sensitive to CL-induced killing [71].

The possibility that cell-type- or species-specific differences could explain the lack of E1B 19 kD blockade of TNF-induced apoptosis in E1A-expressing cells was also tested. The same sets of NIH-3T3 and (10)3 cells tested against CL-induced injury were compared with the BHK-21 cell lines, BHK-21 clone 13.8 (no oncogene expression), BHK-B2 (E1A expression only), and BHK-D5 (E1A+E1B co-expression) [26] (Fig. 7). E1B 19 kD-related differences in TNF-induced apoptosis in these experiments varied with both the cell type and the TNF concentration tested. For example, in comparison studies of NIH-3T3 cells, E1B 19 kD co-expression resulted in a significant reduction in the apoptotic sensitivity of E1A-positive cells at low (6 and 12 ng/ml), but not high (25 and 50 ng/ml) TNF concentrations (Fig. 7A). This result suggested that weak blockade of TNF-induced apoptosis can be detected in vitro, but that this E1B effect is overcome at higher TNF concentrations. Experiments with (10)3 mouse cells and BHK-21 hamster cells showed no blocking effect of E1B 19 kD against TNF-induced apoptosis of E1A-sensitized cells. Indeed, with both of these types of cells, there was a trend toward increased TNF-induced apoptosis with E1A+E1B-expressing cells compared with cells expressing E1A alone (Fig. 7B and 7C).

DISCUSSION

These studies show that the mechanism by which the E1A oncoprotein sensitizes mammalian cells to immune-mediated apoptosis does not require expression of p53 in either rodent fibroblasts (Fig. 1) or human tumor cells (Fig. 2). These results suggest that E1A-induced sensitization to immune-mediated apoptosis should be effective in other rodent or human tumor cells that lack p53 or in which p53 mutations render the antioncogene nonfunctional. In contrast, these data (Table 1) and those of others [54, 55] indicate that triggering of apoptosis by certain chemical injuries can require p53 expression, even if the cells being tested are highly sensitive to p53-independent, proapoptotic injuries.

The CL assays used for these studies were done so that the apoptosis of E1A-positive target cells was caused by degranulation-dependent injury and not by injury caused by CL-expressed Fas ligand [24]. This distinction separates the mechanism of CL-induced injury that was tested here from the triggering mechanism involved in TNF-induced apoptosis, which is analogous to the Fas mechanism [reviewed in 2]. TNF triggering of apoptosis is caused by binding of this cytokine to its cell surface, heterodimeric receptors that activate the caspase cascade through TNF-receptor-associated proteins [reviewed in 2].

The chemical injuries tested were also selected because of the differences in their mechanisms of triggering apoptosis. For example, hygromycin is a protein synthesis inhibitor that likely initiates apoptosis through a mechanism that is completely different from that of either CL or TNF. Although it is not known how these three different types of injuries converge to cause cellular apoptosis, it is clear that none of them require p53 expression to induce apoptosis in E1A-sensitized mouse fibroblasts (Table 1 and Fig. 4A). In contrast, apoptosis induced in E1A-positive cells by three other, diverse types of chemical injuries – beauvericin (potassium ionophore), etoposide (topoisomerase II inhibitor) and H_2O_2 (source of reactive oxygen intermediates) - was strictly p53-dependent (Table 1). The diversity of the injury mechanisms that can trigger apoptosis in E1A-positive cells strongly suggests that the mechanism of E1A-induced sensitization does not involve any one specific type of apoptosis initiating signal.

Instead, these results favor the conclusion that E1A affects a later stage in the cellular response to apoptotic injury.

The marked difference among proapoptotic agents in the p53 requirement to initiate injury of E1A-sensitized cells raises questions about the role of p53 in apoptosis initiation. Perhaps the best known example of the variable role of p53 in the injury-initiation phase of apoptosis is from mouse thymocyte studies [13, 56]. DNA damage of thymocytes caused by ionizing radiation is p53-dependent for inducing apoptosis, whereas glucocorticoids and calcium-dependent activating agents that mimic T-cell receptor engagement induce apoptosis independently of p53 expression. Growth factor withdrawal from mouse lymphoma cells can also require p53 to initiate cellular injury [38]. These data support the conclusion that it is the cellular mechanism of injury initiation that is p53-dependent, not the inherent apoptotic sensitivity of the thymocytes.

We propose this same interpretation of the E1A data presented here. Our results suggest that it is the apoptosis initiation mechanism that either is or is not dependent on p53 expression, not the cellular mechanism through which E1A sensitizes cells to apoptotic injury. This could reconcile apparent differences between our results and the conclusions of previous reports on the role of p53 in E1A sensitization of mouse cells to injury-induced apoptosis. Studies by Lowe and colleagues indicated that E1A sensitization of mouse fibroblasts to apoptosis is dependent on p53 expression [54, 55]. Ionizing radiation, chemotherapeutic agents (including etoposide) and serum withdrawal were used as the proapoptotic stimuli in their experiments. In our experiments, etoposide-induced apoptosis of E1A-positive mouse cells required p53 expression, but the same E1A-positive, p53-negative cells that were resistant to etoposide-induced apoptosis were highly sensitive to apoptosis induced by CL or TNF (Table 2). It is possible that the E1A-positive, p53-negative mouse cells described by Lowe et al. [54, 55] also would have been found to be susceptible to apoptosis if CL and TNF had been tested as proapoptotic injuries. This study comparison underscores the importance of testing a broad range of proapoptotic stimuli, including CL and TNF alpha, when assessing the requirement for p53 in determining the apoptotic sensitivity or resistance of E1A-expressing cells.

Creation of stable, E1A expressing cell lines, such as those used in these studies, involves extensive cell selection. It has been argued that selection of E1A-positive cells during cloning could require collateral, cellular gene mutations [88]. Therefore, it is possible that one or more such mutations is required for the apoptosis sensitive phenotype observed with the E1A-positive cells tested in our studies. However, other data from this laboratory indicate that cell-selection-related mutations are not required for E1A-induced sensitization to apoptotic injury. We have observed that transient expression of E1A oncoproteins, without cell selection, results in exactly the same E1A-induced sensitization to apoptotic injury as is reported here with the stable, E1A expressing cells. In one such experiment, p53-positive NIH-3T3 cells were induced to express E1A following IPTG treatment. These cells exhibited the same E1A-induced sensitization to immune-mediated (Cook, J.L., unpublished data) and chemical [16] apoptotic injuries that was observed in the studies presented here. In a second type of experiment, NIH-3T3 cells transiently transfected with an E1A expression plasmid were highly sensitive to TNF-induced apoptosis within days after transfection and without any cell selection (Cook, J. L., unpublished data). Therefore, it is unlikely that any cellular mutations are required to complement E1A activities to induce sensitization to apoptotic injuries.

There have been other reports of p53-independent, E1A-induced apoptosis. However, most of these observations appear to be related to an indirect effect of E1A, resulting from its activation of an Ad E4 ORF4 gene product which, in turn, induces p53-independent apoptosis through a mechanism that remains to be defined [11, 49, 57, 81, 85]. In another report of TNF-induced, p53-independent apoptosis in which the target cells expressed E1A [47], the cells also expressed a mutant *ras* oncogene that has been reported to variably increase or decrease the cellular apoptotic response [5-7, 34, 35, 45, 61, 91]. Indeed, mutant ras expression itself can cause p53-independent apoptosis [58]. Therefore, it is difficult to assess the independent role of E1A in sensitizing cells to proapoptotic stimuli, when the cells co-express mutant *ras*.

The antiapoptotic activity of E1B 19 kD was either completely or relatively ineffective in blocking the p53-independent, proapoptotic injuries inflicted by CL or TNF on E1A-sensitized cells in these studies (Figs. 4, 6 and 7). These results are consistent with our reported

observations that E1B gene expression does not block E1A sensitization to CL-induced killing of Ad-infected hamster cells, Ad-transformed hamster, rat or human cells, or stably E1A-transfected BHK-21 cells [19, 20, 25, 26, 50, 71, 90]. Others have also reported that E1B co-expression does not block TNF-induced apoptosis of E1A-expressing rat or mouse cells [48, 87].

These observations can be considered in the context of information on the mechanisms by which CL and TNF trigger apoptosis and E1B 19 kD blocks this response to provide insight into possible mechanisms by which E1A sensitizes cells to immune-mediated apoptosis. Degranulation-dependent killing by CL triggers the apoptosis cascade primarily through the actions of granzyme B, which directly activates several cellular caspases, including caspases 8 and 3 [28, 36, 59, 64, 70]. TNF triggering of apoptosis is initiated by its binding and trimerization of the TNF receptor which, in turn, activates the caspase cascade at the level of caspase 8, through an intermediary protein, FADD [reviewed in 2]. Relatively little is known about the mechanisms by which E1B 19 kD blocks apoptosis. However, it is reported that E1B 19 kD interferes with the function of FADD in its activating interactions with caspase 8 [65]. There are also data suggesting that E1A might activate caspase 8 [63] and that the kinetics of cell injury-induced activation of caspase 8 can be cell type-specific [76]. It is apparent from these observations that several molecular interactions in the initiation of the caspase cascade could influence the balance between injury-induced activation of apoptosis, E1A enhancement of this activation, and the ability of E1B to block it. There is also evidence that CL-injury can completely bypass the caspase activation sequence to induce apoptosis [74, 86]. Therefore, it is possible that E1A activates a step in this less well defined, "post-caspase" stage in the cellular apoptotic response. Another possibility is that E1A might sensitize cells to immune-mediated, proapoptotic injuries by reducing the cellular, antiapoptotic defense, rather than by enhancing one or more steps in the caspase cascade or post-caspase apoptosis activation response. Considering the multiple molecular activities that have been linked with E1A expression, it is likely that the final answer regarding the mechanism(s) of E1A-induced cellular sensitization to apoptosis will involve alterations at multiple steps in the cellular response to proapoptotic injury.

These results and previously reported data can be used to consider the implications of E1A-induced sensitization to immune-mediated apoptosis and E1B 19 kD antiapoptotic activity for tumor formation in immunocompetent animals. The results in Figures 1, 2, 4 and 6 and previously published data [25, 26, 71, 90] indicate that E1A expression sensitizes both rodent and human tumor cells to CL-induced apoptosis and that E1B 19 kD expression cannot block this E1A activity. The data in Figure 7 show that the same E1A-induced sensitization and lack of E1B 19 kD blockade occur with TNF-treated hamster sarcoma cells. We reported that Ad-transformed cells that express high levels of both E1A and E1B 19 kD proteins are highly susceptible to killing by activated macrophages [18, 19, 21, 22, 25, 50] which use TNF as a major cytotoxic factor against E1A-expressing cells [23]. We have also observed that hamster, BHK-21 sarcoma cells that co-express E1A+E1B 19 kD proteins are rejected by immunocompetent animals as efficiently as BHK-21 cells that express only E1A [26]. These in vivo data are consistent with many reports that Ad2- and Ad5-transformed rodent cells, most of which are likely to express high levels of both E1A and E1B 19 kD proteins, are unable to form tumors in immunocompetent animals [reviewed in 51] and that E1A expression causes apoptosis in tumor cells in vivo [30]. These data suggest that E1B 19 kD antiapoptotic activity that can be detected in some in vitro assays is not a significant factor in vivo in determining the tumorigenicity of E1A-expressing tumor cells in immunologically intact animals.

Collectively, the observations in this report indicate that the mechanisms by which E1A sensitizes tumor cells to immune-mediated apoptosis and to rejection by the cellular immune response in vivo does not require cellular expression of wild type p53 and can function independently of the Bcl-2-like, antiapoptotic activities of E1B 19 kD. The precise molecular mechanism(s) of E1A-induced, p53-independent sensitization of cells to apoptosis triggered by immune-mediated injuries and other proapoptotic stimuli remain to be defined.

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Table 1. Injury specificity of the p53-dependence of E1A sensitization to apoptosis.

	Target Cells			
Cell Characteristics	NIH-3T3	3T3-E1A	(10)3	(10)3-E1A
p53	+	+	-	-
E1A	-	+	-	+
Injury	**%Apoptosis**			
CL (E:T = 6:1)	12 ± 5	44 ± 4	10 ± 2	45 ± 7
TNF alpha (25 ng/ml)	6 ± 1	42 ± 8	4 ± 3	44 ± 5
Hygromycin (800 ug/ml)	4 ± 2	56 ± 9	18 ± 2	70 ± 8
Beauvericin (12 uM)	1 ± 1	60 ± 9	1 ± 1	2 ± 1
Etoposide (25 uM)	9 ± 4	55 ± 8	7 ± 2	4 ± 1
Hydrogen Peroxide (0.7 mM)	6 ± 1	51 ± 3	1 ± 3	9 ± 3

The apoptosis sensitizing effect of E1A expression on p53-negative, (10)3 cells was compared with the E1A effect on p53-positive, NIH-3T3 cells using a variety of proapoptotic injuries. The percentage of apoptotic cell death was quantitated by radiolabel release. The apoptotic nature of cell death was confirmed by enumeration of apoptotic nuclei. Each set of data represents the mean ± SEM results of at least three experiments. E1A-positive, NIH-3T3 cells were significantly more sensitive than E1A-negative NIH-3T3 cells to apoptosis induced by all six injuries ($p < 0.05$). E1A-positive, (10)3 cells were significantly more sensitive than E1A-negative (10)3 cells to cytolytic lymphocytes (CL), TNF alpha and hygromycin ($p < 0.05$). The differences in the sensitivities of E1A-positive and E1A-negative (10)3 cells to apoptosis induced by beauvericin, etoposide and hydrogen peroxide were not significant ($p > 0.10$).

FIGURE LEGENDS

Figure 1. E1A-induced sensitization of mouse fibroblasts to apoptosis induced by cytolytic lymphocytes (CL) is not affected by the absence of cellular, p53 expression. p53-negative, (10)3 cells were contrasted with p53-positive, NIH-3T3 cells for the ability to exhibit E1A sensitization to CL-induced apoptosis. The mouse CTL clone, 4.1, was used as the source of CL-induced injury in these experiments. Points represent the mean ± SEM results of three experiments in which CL-induced apoptotic cell killing was quantitated at CL:target cell ratios from 1:1 to 12:1. E1A-positive, p53-negative cells were equally susceptible to CL-induced apoptosis as E1A-positive, p53-positive cells ($p > 0.10$). E1A-negative cells of both types were resistant to CL-induced apoptosis. E1A-positive cells of both types were significantly more susceptible to CL-induced apoptosis than were the respective, E1A-negative cells ($p < 0.05$)

Figure 2. E1A sensitizes p53-negative, human tumor cells to NK cell-induced apoptosis. Human Saos-2, osteosarcoma cells stably expressing E1A oncoprotein but lacking the p53 gene were compared with E1A-negative control cells for sensitivity to killer cell-induced apoptosis. Human NK cells were used as the source of CL-induced injury. Points represent the mean ± SEM results of six experiments in which apoptotic killing by human NK cells was quantitated at blood lymphocyte:target cell ratios from 12:1 to 100:1. E1A-positive, Saos-2 cells were significantly more susceptible to NK-cell-induced apoptosis than E1A-negative cells at blood lymphocyte:target cell ratios of 50:1 and 100:1 ($p < 0.05$).

Figure 3. E1A sensitization to TNF-induced apoptosis is independent of p53 expression. The same set of NIH-3T3 mouse target cells tested in the experiments represented in Figure 1 were tested for sensitivity to apoptosis induced by recombinant, mouse TNF-alpha at the indicated TNF concentrations. Points represent the mean ± SEM results of three experiments. E1A expression induced both p53-negative, (10)3 cells and p53-positive, NIH-3T3 cells to become significantly more susceptible to TNF-induced apoptosis than the respective, E1A-negative

control cells ($p < 0.05$). There was no significant difference in the TNF-sensitivity of E1A-positive, p53-negative cells contrasted with E1A-positive, p53-positive cells ($p > 0.10$).

Figure 4. E1B 19 kD protein expression does not block E1A sensitization to immune-mediated apoptosis, but blocks chemically induced apoptosis of E1A-positive cells. NIH 3T3 cells expressing either E1A alone (E1A+ = 3T3-E1A cells) or both the E1A and E1B 19 kD oncoproteins (E1A+, E1B 19 kD+ = E1PB9 cells) were tested under conditions which had been optimized for triggering apoptosis of E1A-positive cells by the six different injuries indicated. The three injuries represented in **A** (CL, TNF and hygromycin) induced apoptosis independently of target cell p53 expression, whereas the three injuries represented in **B** (beauvericin, etoposide and H_20_2) required p53 expression to induce apoptosis (Table 1). Bars represent the mean ± SEM results of three or four experiments in which each injury was tested against all three cell types. E1A-positive cells were significantly more sensitive to apoptotic death caused by all six injuries than E1A-negative cells ($p < 0.05$). E1B 19 kD protein co-expression had no inhibitory effect on the sensitivity of E1A-positive cells injured with the two immune mediators of apoptosis, CL and TNF-alpha ($p > 0.10$). In contrast, E1B 19 kD co-expression caused a significant reduction in the ability of all four chemical injuries to induce apoptosis in E1A-positive cells ($p < 0.05$), resulting in a reduced level of apoptosis sensitivity that was not significantly greater than that exhibited by E1A-negative, NIH-3T3 cells ($p > 0.10$).

Figure 5. NIH-3T3 cells selected for studies of the effects of E1A 19 kD protein expression on E1A sensitization to injury-induced apoptosis express a high level of E1B 19 kD oncoprotein. Quantitative immunoblot analysis of E1B 19 kD protein expression in an NIH-3T3 cell clone (E1PB9) selected for co-expression of E1A and E1B contrasted with E1B 19 kD expression in 293 cells which were known to stably express E1B 19 kD at a high level. Nontransfected NIH-3T3 cells and NIH-3T3 cells transfected with only the E1A gene (3T3-E1A) served as E1B-negative controls.

Figure 6. E1B 19 kD co-expression does not block CL-induced apoptosis of either E1A-expressing NIH-3T3 cells or E1A-expressing (10)3 cells across an eight fold range of killer cell to target cell ratios. NIH-3T3 **(A)** and (10)3 **(B)** cells expressing either E1A alone or E1A+E1B 19 kD were compared with nontransfected parental cells for sensitivity to CL-induced apoptosis using the mouse CTL clone, 4.1, as the source of CL-induced injury. Points represent the mean ± SEM of three experiments with each set of target cells. The sensitivity to CL-induced apoptosis of NIH-3T3 or (10)3 cells co-expressing E1A+E1B 19 kD was not significantly different from that of the cells of the same type expressing E1A alone. Both NIH-3T3 and (10)3 cells expressing either E1A alone or E1A+E1B 19 kD proteins were significantly more sensitive to CL-induced apoptosis than the respective, E1A-negative parental cells ($p < 0.05$).

Figure 7. E1B 19 kD co-expression had a variable effect on TNF-induced apoptosis of E1A-positive cells mouse and hamster cells that was cell type-specific and was eliminated at high TNF concentrations. NIH-3T3 cells **(A)**, (10)3 cells **(B)** (both mouse fibroblast cell lines) and BHK-21 hamster cells **(C)** expressing E1A alone or co-expressing E1A+E1B 19 kD were tested for sensitivity to apoptosis induced by recombinant, mouse TNF at the indicated TNF concentrations. Points represent the mean ± SEM results of five **(A)**, six **(B)**, or four **(C)** experiments. There was a TNF -concentration-dependent effect of E1B 19 kD expression on TNF-induced apoptosis of E1A-positive mouse cells. **(A)** At low TNF concentrations (6 and 12 ng/ml) E1B 19 kD co-expression was associated with *reduced* sensitivity of E1A-positive, NIH-3T3 cells ($p < 0.05$), whereas at high TNF concentrations (25 and 50 ng/ml), there was no significant difference in the TNF sensitivity of cells expressing E1A+E1B 19 kD vs. E1A alone. **(B)** The results with (10)3 cells were opposite to those with NIH-3T3 cells at low TNF concentrations where E1B 19 kD co-expression was associated with *increased* sensitivity of E1A-positive cells to TNF-induced apoptosis ($p < 0.05$). As had been observed with the NIH-3T3 lines, there was no significant difference in the sensitivity of (10)3 cells expressing E1A+E1B 19 kD vs. E1A alone at high concentrations of TNF. **(C)** In contrast to the results with the two mouse cell lines, there was no significant difference in the sensitivity of BHK-21 cells to

TNF-induced apoptosis resulting from E1B 19 kD co-expression with E1A vs. cells expressing E1A alone. There was a consistent trend toward increased TNF sensitivity of the cells co-expressing E1A+E1B kD, however. At all TNF concentrations tested, cells of all three types that either expressed E1A alone or E1A+E1B 19 kD proteins were significantly more sensitive to TNF-induced apoptosis than the respective, nontransfected control cells ($p < 0.05$).

Fig. 1

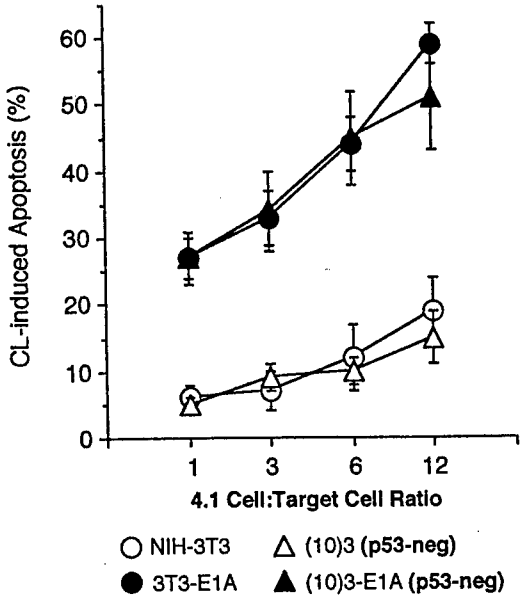


Fig. 3

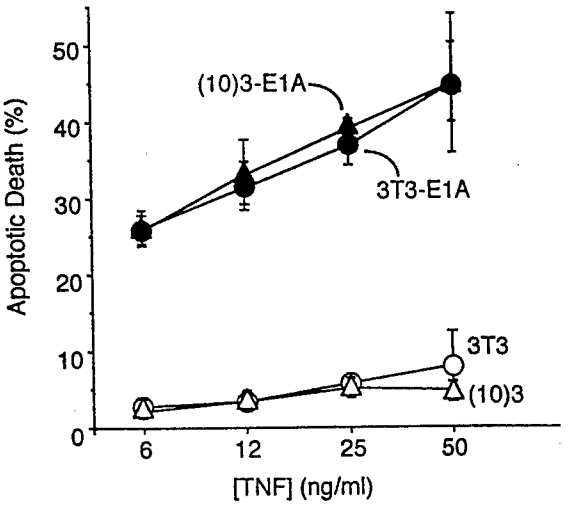
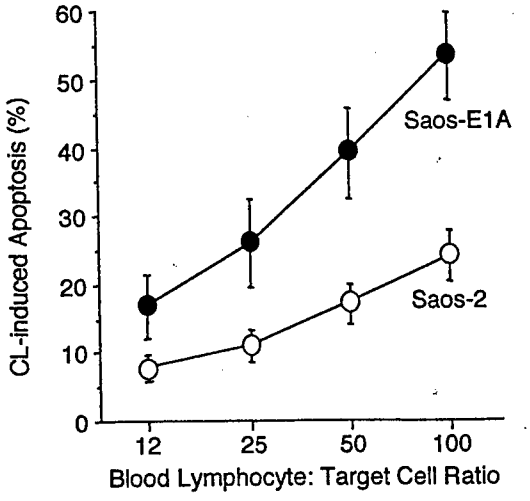


Fig. 2



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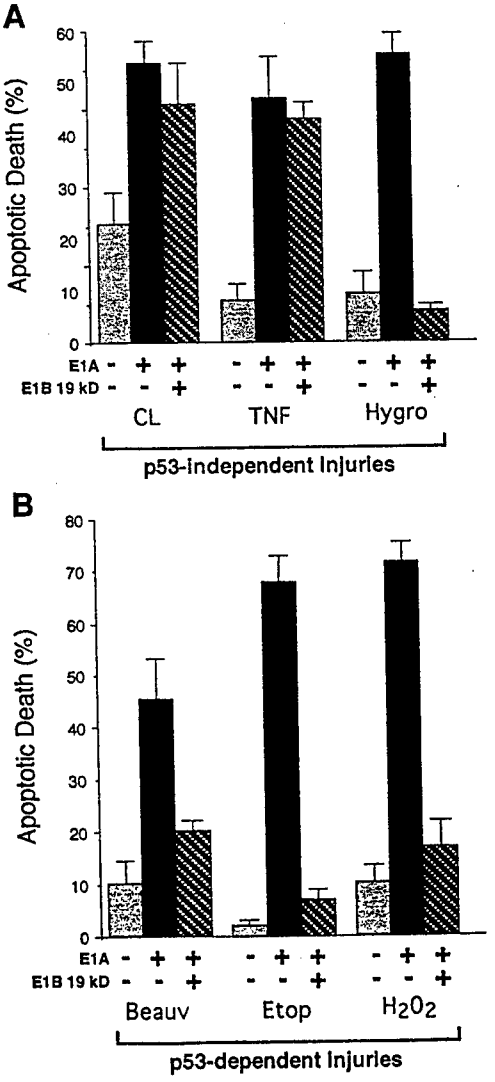


Fig. 5

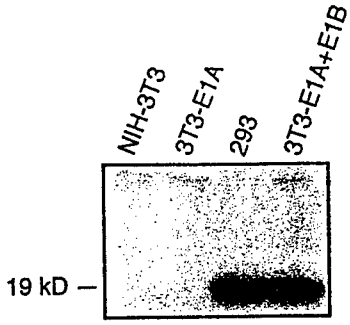
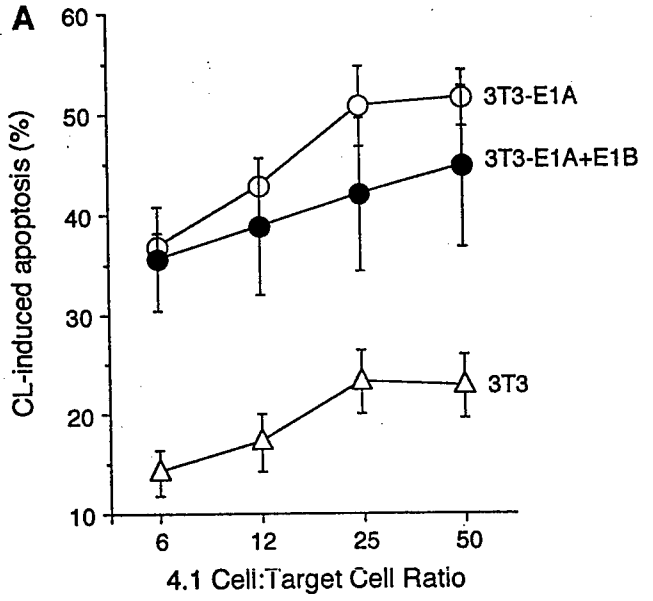


Fig. 6



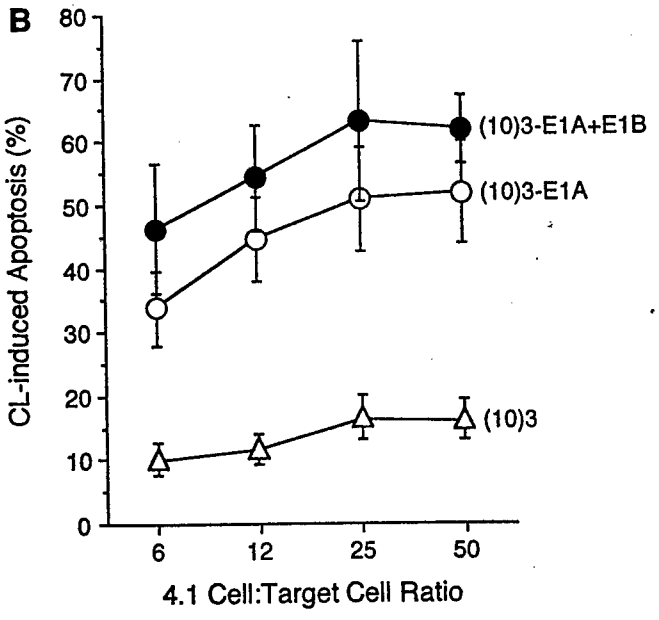
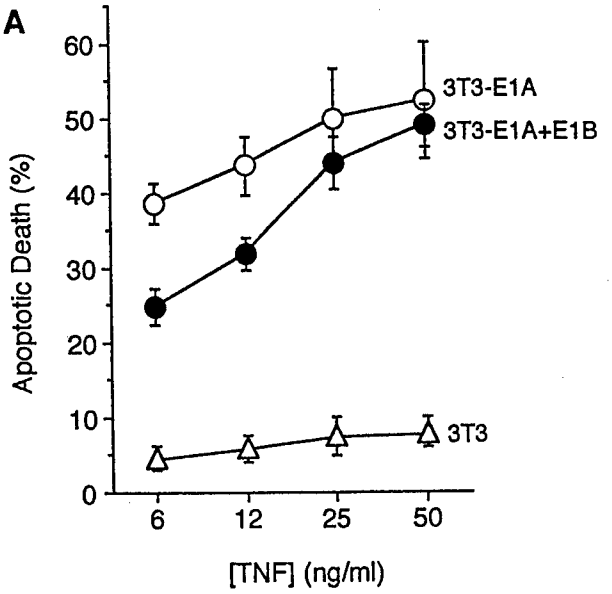
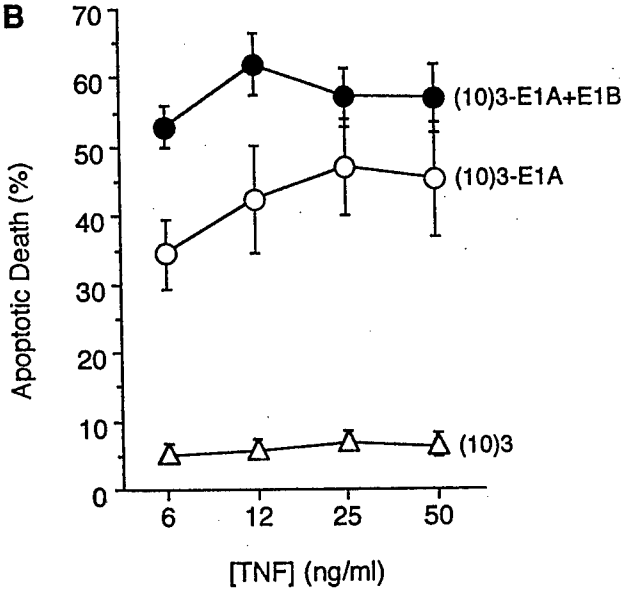
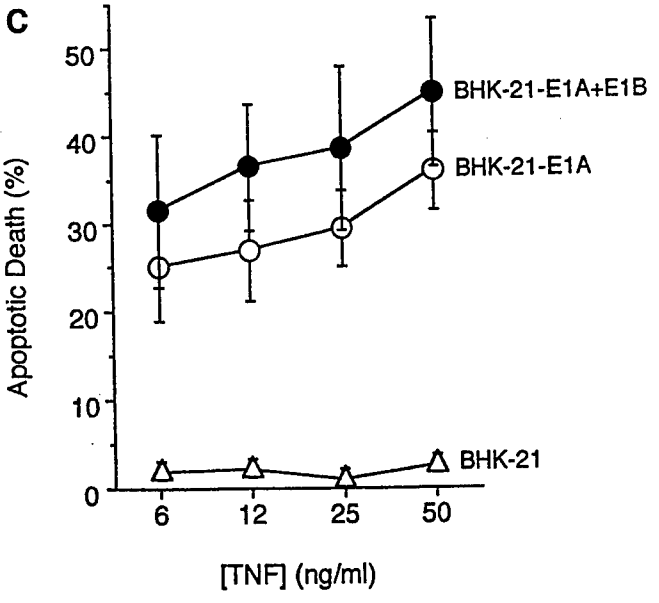


Fig. 7







DEPARTMENT OF THE ARMY
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